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## (57) Abstract

The present invention provides novel methods for identifying and characterizing compounds that modulate the activity of an LPA receptor.

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## METHODS FOR DETECTING COMPOUNDS WHICH MODULATE THE ACTIVITY OF AN LPA RECEPTOR

### TECHNICAL FIELD

5           The present invention relates to methods for identifying and characterizing  
compounds that modulate the activity of a member of the EDG family of receptors, including  
EDG-1, EDG-2, EDG-3, EDG-4, and EDG-5 (gene sequences and amino acid sequences  
represented by SEQ ID NOS: 1, 4, 5, 6 and 7, respectively), and a similar receptor called  
10       PSP-24 (gene sequence and amino acid sequence represented by SEQ ID NOS: 8 and 9,  
respectively.)

### BACKGROUND

Cellular signal transduction is a fundamental mechanism whereby external stimuli  
that regulate diverse cellular processes are relayed to the interior of cells. Frequently, binding  
of a ligand to a cell-surface receptor represents the first step in a cascade of events that results  
15       in a cellular response. The ligands recognized by specific receptors include a diverse array of  
molecules such as peptides, deoxyribonucleotide triphosphates and phospholipids.

Research into phospholipid signaling is an area of intense scientific investigation, as  
more and more bioactive lipids are being identified and their actions characterized. One  
important addition to the growing list of lipid messengers is lysophosphatidic acid (1-acyl-2-  
20       hydroxy-*sn*-glycero-3-phosphate, LPA), the simplest of all glycerophospholipids. While  
LPA has long been known as a precursor of phospholipid biosynthesis in both eukaryotic and  
prokaryotic cells, only recently has LPA emerged as an intercellular signaling molecule that  
is rapidly produced and released by activated cells, notably platelets, to influence target cells  
by acting on a specific cell-surface receptor. Moolenaar (1994) *Trends Cell Biol.* 4:213-219.  
25       Besides being synthesized and processed to more complex phospholipids in the endoplasmic  
reticulum, LPA can be generated through the hydrolysis of pre-existing phospholipids  
following cell activation. The best documented example concerns thrombin-activated  
platelets, where LPA production is followed by its extracellular release. Eichholtz et al.  
(1993) *Biochem. J.* 291:677-680. Platelet LPA is formed, at least in large part, through  
30       phospholipase A<sub>2</sub> (PLA<sub>2</sub>)-mediated deacylation of newly generated phosphatidic acid (PA).  
Gerrard and Robinson (1989) *Biochim. Biophys. Acta* 1001:282-285. Distinct PA-specific  
PLA<sub>2</sub> activity has been identified in platelets (Ca<sup>2+</sup>-dependent) and in rat brain (Ca<sup>2+</sup>-

independent), but little is known about its mode of regulation. Billah et al. (1981) *J. Biol. Chem.* 256:5399-5403; and Thompson and Clark (1995) *Biochem. J.* 306:305-309.

It remains to be examined at what stage of the platelet activation response LPA is produced and how it is released into the extracellular environment. Given the wide variety of LPA responsive cell types, LPA production and release are unlikely to be restricted to platelets. Indeed, there is preliminary evidence that growth factor-stimulated fibroblasts can also produce LPA. Fukami and Takenawa (1992) *J. Biol. Chem.* 267:10988-10993. Furthermore, LPA may be formed and released by injured cells, probably due to nonspecific activation of phospholipases. Many other cell systems remain to be examined for LPA production.

In freshly prepared mammalian serum, LPA concentrations are estimated to be in the range of approximately 2-20  $\mu$ M, with oleoyl- and palmitoyl-LPA being the predominant species. Tokumura et al. (1994) *Am. J. Physiol.* 267:C204-C210; and Eichholtz et al. (1993) *Biochem. J.* 291:677-680. LPA is not detectable in platelet-poor plasma, whole blood, or cerebrospinal fluid. Tigyi and Miledi (1992) *J. Biol. Chem.* 267:21360-21367. In common with long chain fatty acids, LPA binds with high affinity to serum albumin at a molar ratio of about 3:1. Tigyi et al. (1991) *J. Biol. Chem.* 266:20602-20609; Thumser et al. (1994) *Biochem. J.* 301:801-806. It is notable that serum albumin contains several other, as yet unidentified lipids (methanol-extractable) with LPA-like biological activity. Tigyi and Miledi (1992) *J. Biol. Chem.* 267:21360-21367. This raises the interesting possibility that LPA may belong to a new family of phospholipid mediators showing overlapping biological activities and acting on distinct receptors; conceivably, the ether-linked phospholipid platelet-activating factor (PAF) and the mitogenic lipid sphingosine 1-phosphate may also belong to this putative family. Zhang et al. (1991) *J. Cell Biol.* 114:155-167.

The range of biological responses to LPA is quite diverse, ranging from induction of cell proliferation to stimulation of neurite retraction and even slimemold chemotaxis, and the body of knowledge continues to grow as more and more cellular systems are tested for LPA responsiveness. Jalink et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:1857-1861; Jalink et al. (1993) *Cell Growth and Differ.* 4:247-255; and Moolenaar (1995) *Curr. Opin. Cell Biol.* 7:203-210; Dyer et al. (1992) *Molec. Brain Res.* 14:293-301; Dyer et al. (1992) *Molec. Brain Res.* 14:302-309; Tigyi and Miledi (1992) *J. Biol. Chem.* 267:21360-21367.

Although its precise physiological and pathological functions *in vivo* remain to be explored, LPA derived from platelets has all the hallmarks of an important mediator of wound healing and tissue regeneration. Thus, in addition to acting as an autocrine stimulator of platelet aggregation, LPA stimulates the growth of fibroblasts, vascular smooth muscle cells, endothelial cells, and keratinocytes. Moolenaar (1994) *Trends Cell Biol.* 4:213-219; Jalink et al. (1994) *Biochim. Biophys. Acta* 1198:185-196; Van Corven et al. (1989) *Cell* 59:45-54; Tigyi et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:1908-1912; Tokumura et al. (1994) *Am. J. Physiol.* 267:C204-C210; and Piazza et al. (1995) *Exp. Cell Res.* 216:51-64. Intriguingly, it has been observed that LPA acts as an inhibitor of eukaryotic DNA polymerase  $\alpha$ . Murakami-Murofushi et al. (1992) *J. Biol. Chem.* 267:21512-21517. LPA also exhibits anti-mitogenic activity toward myeloma cells, presumably through a distinct receptor subtype. Tigyi et al. (1994) *Proc. Natl. Acad. Sci.* 91:1908-1912; Murakami-Murofushi et al. (1993) *Cell Structure and Function* 18:363-370.

In addition to stimulating cell growth and proliferation, LPA promotes cellular tension and cell-surface fibronectin binding, which are important events in wound repair and regeneration. Zhang et al. (1994) *J. Cell Biol.* 127:1447-1459; Kolodney et al. (1993) *J. Biol. Chem.* 268:23850-23855; and Lapetina et al. (1981) *J. Biochem.* 256:5037-5040. As a product of the blood-clotting process, LPA is a normal constituent of serum (but not platelet-poor plasma), where it is present in an albumin-bound form at physiologically relevant concentrations. Tigyi and Miledi (1992) *J. Biol. Chem.* 267:21360-21367; and Eichholtz et al. (1993) *Biochem. J.* 291:677-680.

Recently, anti-apoptotic activity has also been ascribed to LPA. PCT Application No. PCT/US94/13649. In this study, an actively proliferating cell line was rescued from serum withdrawal-induced apoptosis by LPA. In another study, evidence has been presented suggesting that LPA can suppress apoptosis *in vitro* as well as in ischemic organs such as heart and liver. Wu et al. (1996) *Transplantation* (in press).

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents,

ionizing radiation, and infection by human immunodeficiency virus (HIV). Wyllie (1980) *Nature* 284:555-556; Kanter et al. (1984) *Biochem. Biophys. Res. Commun.* 118:392-399; Duke and Cohen (1986) *Lymphokine Res.* 5:289-299; Tomei et al. (1988) *Biochem. Biophys. Res. Commun.* 155:324-331; Kruman et al. (1991) *J. Cell. Physiol.* 148:267-273; Ameisen and Capron (1991) *Immunol. Today* 12:102-105; and Sheppard and Ascher (1992) *J. AIDS* 5:143-147. Agents that affect the biological control of apoptosis thus have therapeutic utility in numerous clinical indications.

Cellular shrinkage, chromatin condensation, cytoplasmic blebbing, increased membrane permeability and interchromosomal DNA cleavage characterize Apoptotic cell death. Gerschenson et al. (1992) *FASEB J.* 6:2450-2455; and Cohen and Duke (1992) *Ann. Rev. Immunol.* 10:267-293. The blebs, small, membrane-encapsulated spheres that pinch off of the surface of apoptotic cells, may continue to produce superoxide radicals which damage surrounding cell tissue and may be involved in inflammatory processes.

While apoptosis is a normal cellular event, pathological conditions and a variety of injuries can also induce it. Apoptosis is involved in a wide variety of conditions, including, but not limited to, cardiovascular disease; cancer regression; immune disorders, including, but not limited to, systemic lupus erythematosus; viral diseases; anemia; neurological disorders; diabetes; hair loss; rejection of organ transplants; prostate hypertrophy; obesity; ocular disorders; stress; aging; and gastrointestinal disorders, including, but not limited to, diarrhea and dysentery. In the myocardium, apoptotic cell death follows ischemia and reperfusion.

In Alzheimer's disease, Parkinson's disease, Huntington's chorea, epilepsy, amyotrophic lateral sclerosis, stroke, ischemic heart disease, spinal cord injury and many viral infections, for example, abnormally high levels of cell death occur. In at least some of these diseases, there is evidence that the excessive cell death occurs through mechanisms consistent with apoptosis. Among these are 1) spinal cord injury, where the severing of axons deprives neurons of neurotrophic factors necessary to sustain cellular viability; 2) stroke, where after an initial phase of necrotic cell death due to ischemia, the rupture of dead cells releases excitatory neurotransmitters such as glutamate and oxygen free radicals that stimulate apoptosis in neighboring healthy neurons; and 3) HIV infection, which induces apoptosis of T-lymphocytes.

In contrast, the level of apoptosis is decreased in cancer cells, which allows the cancer cells to survive longer than their normal cell counterparts. As a result of the increased

number of surviving cancer cells, the mass of a tumor can increase even if the doubling time of the cancer cells does not increase. Furthermore, the high level of expression in a cancer cell of the bcl-2 gene, which is involved in regulating apoptosis and, in some cases, necrotic cell death, renders the cancer cell relatively resistant to chemotherapeutic agents and to radiation therapy.

There is considerable evidence of plasma membrane receptors for LPA. LPA-binding proteins have been reported in mammalian tissues and labeled using a photoaffinity crosslinker derivative. Liliom et al. (1996) *Am. J. Physiol.* 270:C772-C778; Thomson et al. (1994) *Mol. Pharmacol.* 45:718-723; and van der Bend et al. (1992) *EMBO J.* 11:2495-2501. In *X. laevis* oocytes, LPA elicits oscillatory  $Cl^-$  currents. Tigyi and Miledi (1992) *J. Biol. Chem.* 267:21360-21367. This current, like other effects of LPA, is consistent with a plasma membrane receptor-mediated activation of G protein-linked signal transduction pathways.

G proteins are comprised of three subunits: a guanyl-nucleotide binding  $\alpha$  subunit; a  $\beta$  subunit; and a  $\gamma$  subunit. G proteins cycle between two forms, depending on whether GDP or GTP is bound thereto. When GDP is bound the  $G_{\alpha\beta\gamma}$  protein exists as an inactive heterotrimer, the  $G_{\alpha\beta\gamma}$  complex. When GTP is bound the  $\alpha$  subunit dissociates, leaving a  $G_{\beta\gamma}$  complex. Importantly, when a  $G_{\alpha\beta\gamma}$  complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of dissociation of the bound the  $\alpha$  subunit from the  $G_{\beta\gamma}$  complex increases. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena.

At least four G protein-mediated signaling pathways have been identified in the action of LPA. These are: 1) stimulation of phospholipase C and phospholipase D; 2) inhibition of adenylyl cyclase; 3) activation of Ras and the downstream Raf/MAP kinase pathway; and 4) tyrosine phosphorylation of focal adhesion proteins in concert with remodeling of the actin cytoskeleton in a Rho-dependent manner.

GTP-binding proteins fall into two broad classes of regulatory proteins; the heterotrimeric G-proteins, and small GTPases. The alpha subunit of heterotrimeric G-proteins ( $G_{\alpha}$ ) and the small GTPases, as typified by the proto-oncogene Ras, share certain structural homology, and cycle between an active GTP-bound state and an inactive GDP-bound state. When stimulated by an appropriate signal, G-proteins and small GTPases become activated by the binding of GTP and physically interact with effector molecules to

transduce the signal to the cell. In the case of G-proteins, binding of GTP to the  $\alpha$  subunit causes the low molecular weight  $G\alpha$  to dissociate from the  $G\beta\gamma$  dimer where either  $G\alpha$  or  $G\beta\gamma$  can act as the signal transducer. An intrinsic GTPase activity hydrolyses GTP to GDP and thus attenuates the signal. Ancillary proteins collectively known as exchange factors are responsible for replacing GDP for GTP and reactivating the GTP-binding protein.

Heterotrimeric G-protein coupled receptors are a special class of receptors. It is estimated that G-protein coupled receptors comprise 0.1% of the human genome (including olfactory and visual receptors) which could place the number of different receptors in the thousands. The common structural feature of these receptors are seven hydrophilic membrane spanning domains. Based on the three dimensional model of bacterial rhodopsin, it is predicted that the receptors would form a barrel shaped structure with the ligand binding domains being the extracellular loops and/or the transmembrane domains.

Recently, three putative receptors for LPA have been identified suggesting that functionally different LPA receptors may exist that dictate the particular cellular response of LPA. Hecht, J. H., et al. (1996) J. Cell. Biol. 135(4), 1071-1083; Macrae, A. D., et al. (1996) Mol. Brain. Res. 42, 245-254; An, S., et al. (1997) Biochim. Biophys. Res. Com. 231, 619-622; Guo, Z., et al. (1996) Proc. Natl. Acad. Sci. USA 93, 14367-14372; An, et al., J. Biol. Chem. (1998). Most cell types respond to LPA making it difficult to characterize the receptor dependency of a particular response to LPA since the response cannot be solely attributed to a single LPA receptor. In particular, it is difficult to assess ligand binding specificity of an LPA receptor without a naive cell line because other lipid receptors may exist with overlapping ligand specificity. Therefore, the yeast *Saccharomyces cerevisiae* was used to study the human LPA receptor EDG-2 (or Vzgr-1). *S. cerevisiae* contain no endogenous LPA receptors and is therefore a potentially useful organism in which to functionally express LPA receptors and analyze their ligand specificity. Other mammalian receptors have been functionally expressed in *Saccharomyces* including the sommatostatin receptor. (Price, L. A., et al. (1995) Mol. Cell. Biol. 15(11), 6188-6195), the A2a adenosine receptor (Price, L. A., et al. (1996) Mol. Pharmacol. 50(4), 829-837) and the  $\beta$ 2-adrenergic receptor (King, K., et al., (1990) Science 250, 121-123).

Figure 1 shows a detailed schematic of the yeast pheromone-inducible MAP Kinase cascade. *Saccharomyces* contains a single heterotrimeric G-protein that is activated by mating factor binding to a specific receptor. Blumer, K. J., and Thorner, J. (1990) Proc. Natl. Acad. Sci. USA 87, 4363-4367. Upon stimulation by an occupied receptor, the  $\alpha$



subunit of the heterotrimeric G protein ( $G\alpha$ , the *GPA1* gene product (Dietzel, C., and Kurjan, J. (1987) *Cell* 50, 1001-1010; Miyajima, I., et al. (1987) *Cell* 50, 1011-1019) becomes bound to GTP and dissociates from the  $\beta\gamma$  dimer. In yeast, it is the  $\beta\gamma$  dimer that transduces the signal to Ste11 (the MEKK equivalent (Lange-Carter, C. A., et al. (1993) *Science* 260, 315-319)) and Ste7 (the MEK equivalent (Neiman, A. M., and Herskowitz, I. (1994) *Proc. Natl. Acad. Sci. USA* 91, 3398-3402)). The active GTP-bound version of  $G\alpha$  is inactivated by hydrolysis of GTP to GDP at which time,  $G\alpha$  can re-associate with  $G\beta\gamma$  and attenuates the signal (Blinder, D., and Jenness, D. D. (1989) *Mol. Cell. Biol.* 9, 3720-3726; Cole, G., (1990) *Mol. Cell. Biol.* 10(510-517); Dietzel, C., and Kurjan, J. (1987) *Cell* 50, 1001-1010; Miyajima, I., et al. (1987) *Cell* 50, 1011-1019). Like the mammalian MAP kinase, the yeast MAP kinases Fus1 and Kss1 activate a transcriptional activator, the STE12 gene product (Elion, E. A., et al. (1994) *Mol. Biol. Cell* 4, 495-510). Activated Ste12 in turn activates the transcription of several mating-inducible genes such as *FUS1* (Elion, E. A., et al. (1991) *Cold Spring Harbor Symp. Quant. Biol.* 56, 41-49; Peter, M., et al. (1993) *Cell* 73, 747-760). To study the EDG-2 receptor using the yeast pheromone response pathway system, a strain carrying a mutation in the *FAR1* gene was used. This mutation has the effect of uncoupling the MAP kinase cascade from cell cycle arrest allowing the yeast to continue growing during MAP kinase activation (Peter, M., et al. (1993) *Cell* 73, 747-760; Peter, M., and Herskowitz, I. (1994) *Science* 265, 1228-1231). Secondly, a mutationally inactivated *SST2* gene was created to increase the sensitivity of the strain to G-protein activation. The *SST2* gene encodes a GTPase activating protein (GAP) for the  $G\alpha$  subunit (the *GPA1* gene product) (Dohlman, H. G., et al. (1996) *Mol. Cell. Biol.* 16(9), 5194-5209). By inactivating the *SST2* gene product,  $G\alpha$  remains in the GTP-bound state longer and thus increases the steady-state concentration of the signal transducing  $\beta\gamma$  dimer. Finally, to quantify the response, the bacterial *lacZ* gene was fused to the mating inducible *FUS1* promoter to create a reporter gene.

The ubiquitous presence of the response elicited by LPA in almost every cell line tested, combined with the amphiphilic character of LPA that makes radioligand binding assays extremely difficult, has presented considerable difficulties in the molecular cloning of its receptors. In view of the potential physiological significance of LPA receptors in terms of wound healing, cell regeneration and cell proliferation and apoptosis, there is a need for drug screening assays exhibiting increased specificity that facilitate the search for agonists, inverse

agonists, or antagonists of LPA, as well as methods for screening analogues of LPA to determine their ability to activate EDG-2, for elucidating the pharmacological properties of these proteins.

5 The present invention addresses this need. Herein are described methods of screening for agonists or antagonists of EDG-1, EDG-2, EDG-3, EDG-4, and EDG-5, as well as methods of counter screening for agonists or antagonists that are specific for only one of these EDG receptors.

All references cited herein are incorporated by reference in their entirety.

### SUMMARY OF THE INVENTION

10 Methods of screening for pharmaceutical agents that stimulate, as well as pharmaceutical agents that inhibit, EDG-1, EDG-2, EDG-3, EDG-4, and EDG-5 activity are provided.

15 The present invention encompasses a method for identifying compounds which modulate the activity of any of the EDG receptors, comprising the steps of: a) contacting recombinant host cells, modified to contain the DNA of SEQ. ID. NO. 1, 4, 5, 6, 7 or 8, which is operably linked to control sequences for expression, with at least one compound or signal whose ability to modulate the activity of the EDG receptor is sought to be determined, and b) analyzing the cells for a difference in functional response mediated by said receptor. More specifically, the present invention encompasses contacting said cells with at least one  
20 composition whose ability to modulate the activity of said receptor is sought to be determined, and monitoring said cells for a change in the level of a particular signal associated with activation of the EDG receptor. EDG receptors encompassed by the present invention include EDG-1, EDG-2, EDG-3, EDG-4, and EDG-5. An additional receptor encompassed by the present invention is PSP-24, a receptor of LPA discovered in mice, which can be used as a screen to evaluate the specificity of a particular ligand for any of the  
25 EDG family of receptors. For purposes of the present discussion, PSP-24 shall be encompassed by the expressions "EDG family of receptors" and "EDG related receptors, because it has similarities, including being an LPA receptor.

30 Additionally, the present invention contemplates a method for modulating the signal transduction activity of the EDG receptor, comprising contacting said receptor with an effective amount of at least one compound identified by the method described above.

The present invention also encompasses an agonist, antagonist, inverse agonist, or allosteric modulator identified by the above methods.

In an alternative embodiment, the present invention encompasses a method for detecting an agonist, antagonist, inverse agonist, or allosteric modulator of an EDG receptor having activity comprising the steps of: a) exposing a compound to an EDG receptor coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with the EDG receptor and an associated response through the pathway, and b) detecting an increase or a decrease in the stimulation of the response pathway resulting from the interaction of the compound with the EDG receptor, relative to the absence of the tested compound and therefrom determining the presence of an agonist, antagonist, inverse agonist, or allosteric modulator.

In yet another embodiment, the present invention encompasses a method for detecting an LPA agonist, antagonist, inverse agonist, or allosteric modulator of LPA receptor comprising the steps of a) exposing a compound to the EDG-2 receptor coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with the EDG-2 receptor and an associated response through the pathway, and b) detecting an increase or a decrease in the stimulation of the response pathway resulting from the interaction of the compound with the EDG-2 receptor, relative to the absence of the tested compound and therefrom determining the presence of an agonist, antagonist, inverse agonist, or allosteric modulator.

In yet another embodiment, the invention encompasses a method for detecting inverse agonists of LPA, comprising the steps of a) exposing a compound and LPA to the EDG-2 receptor coupled to a response pathway, under conditions and for a time sufficient to allow interaction of LPA with the EDG-2 receptor and an associated response through the pathway, and b) detecting an increase or a decrease in the stimulation of the response pathway, relative to the absence of the tested compound and therefrom determining the presence of an inverse agonist of LPA.

In yet another embodiment of the present invention, a method of detecting compounds that modulate the interaction between a ligand of an EDG related receptor and the EDG related receptor is encompassed, comprising: exposing a labeled ligand of an EDG related receptor to a cell expressing said EDG related receptor; exposing a labeled compound that is believed to interact with an EDG related receptor to said cell, and detecting a change in the amount of labeled ligand bound to said cell.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of the yeast pheromone-inducible MAP Kinase cascade. Components of this pathway (SST2 and FAR1) that were genetically inactivated by mutation are identified by underlines.

Figure 2 is a graph depicting EDG-2-mediated stimulation of *FUS1::lacZ*. Yeast cells carrying the galactose-inducible *edg-2* gene were grown in SC media containing either 2% galactose (filled bars) or 2% glucose (hatched bars) for seven hours in the presence of lysophosphatidic acid (LPA) or on galactose in the absence of LPA (open bars). After seven hours, the cells were assayed for  $\beta$ -galactosidase (*lacZ*) activity.

Figures 3A and 3B are graphs depicting the stimulation by LPA of *FUS1::lacZ* activity in cells expressing EDG-2 in a time and dose dependent manner, respectively. 3A: Yeast cells carrying *edg-2* (■) or empty vector (◆) were grown in synthetic complete media (S.C.) + 2% galactose for the indicated time prior to assaying  $\beta$ -galactosidase activity. 3B, Yeast cells carrying *edg-2* were grown for seven hours at the indicated dose of LPA.

Figure 4 is a graph depicting the specific activation by LPA, but not other related lysophospholipids or Sphingosine-1-phosphate (SPP), of *FUS1::lacZ*. Yeast cells carrying *edg-2* were grown in S.C. + 2% galactose in the presence of LPA (■), LPC (◆), LPE (●), LPG (▲), LPS (□) or Sph-1-P (○) at the indicated concentrations for seven hours. All lyso-glycerophospholipid were resuspended in BBS/EDTA + 1mg/ml fatty acid free bovine serum albumin to enhance solubility.

Figure 5 is a graph depicting the specific activation of *FUS1::lacZ* by LPA, but not Diacyl-glycerophospholipids. Yeast cells were cultured in S.C. + 2% galactose in the presence of PA (■), PC (◆), PE (●), PG (▲) PS (□) or LPA (○) at the indicated concentration for seven hours. All diacyl-glycerophospholipid were resuspended in BBS/EDTA + 1mg/ml fatty acid free bovine serum albumin to enhance solubility.

Figure 6 is a graph depicting the effect of the fatty acid side-chain of LPA on activation of *FUS1::lacZ*. Yeast cells expressing EDG-2 were cultured in S.C. + 2% galactose in the presence of 18:1 oleoyl LPA (■), 18:0 steroyl LPA (◆), 16:0 palmitoyl LPA (●) or 14:0 (▲) at the indicated concentration for seven hours. The numerical representation refers to the chain length and degree of saturation. All forms of LPA were resuspended in BBS/EDTA + 1mg/ml fatty acid free bovine serum albumin to enhance solubility.

Figure 7 is a graph depicting the activation of *FUS1::lacZ* by LPA presented either as a liposomal formulation or as freely soluble LPA. Yeast cells carrying EDG-2 were culture in S.C. + 2% galactose in the presence of freely soluble LPA (■), LPA + PC liposomes (◆), PC alone (○), LPA + PG liposomes (▲) or PG alone (□). Note that the concentration of lipid reflects only the LPA component of the liposome.

#### MODES FOR CARRYING OUT THE INVENTION

The present invention encompasses methods for detecting substances that modulate EDG receptor activity. Additionally, the present invention encompasses compositions comprising a host organism that does not contain an endogenous LPA receptor and that has been transfected with an EDG receptor. EDG receptors encompassed by the present invention include EDG-1, EDG-2, EDG-3, EDG-4, and EDG-5. An additional receptor encompassed by the present invention is PSP-24, which can be used as a screen to evaluate the specificity of a particular ligand for any of the EDG family of receptors.

The *edg-2* gene product, a lysophosphatidic acid (LPA) receptor, also reported as *vzg-1*, couples to the yeast heterotrimeric G-protein and activates a MAP kinase cascade-dependent reporter. The response to LPA can be quantitated by using a reporter gene, including, without limitation, the *lacZ* gene or the *luc* gene fused to the *FUS1* promoter, a mating pheromone-inducible gene promoter, the *HIS3* gene, or any other gene that can genetically complement an auxotrophic growth mutation. The yeast strain used is able to grow in the presence of activated G-protein due to a mutation in the *FAR1* gene. This mutation has the phenotypic effect of uncoupling G-protein/map kinase activation from cell cycle arrest.

The following definitions are for the purpose of clarifying the terms used herein, and are not meant to be limiting.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector may replicate and function independently of the host genome, as in the case of a plasmid, or may integrate into the genome itself, as in the case of an integratable DNA fragment. Suitable vectors will contain replicon and control sequences that are derived from species compatible with the intended expression host. For example, a promoter operable in a host cell is one which binds the RNA polymerase of that cell, and a ribosomal binding site operable in a host cell is one which binds the endogenous ribosomes of that cell.

DNA regions are "operably" associated when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

5 Heterologous DNA sequences are expressed in a host by means of an expression vector. An expression vector is a replicable DNA construct in which a DNA sequence encoding the heterologous DNA sequence is operably linked to suitable control sequences capable of effecting the expression of a protein or protein subunit coded for by the heterologous DNA sequence in the intended host. Generally, control sequences include a  
10 transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and (optionally) sequences which control the termination of transcription and translation.

Transformed host cells of the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express  
15 the protein or protein subunit coded for by the heterologous DNA sequences.

A variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See, e.g., U.S. Pat. No. 4,745,057; U.S. Pat. No. 4,797,359; U.S. Pat. No. 4,615,974; U.S. Pat. No. 4,880,734; U.S. Pat. No. 4,711,844; and U.S. Pat. No. 4,865,989. *Saccharomyces cerevisiae* is the most commonly used among the yeast, although a number of  
20 other strains are commonly available. See, e.g., U.S. Pat. No. 4,806,472 (*Kluveromyces lactis* and expression vectors therefor); U.S. Pat. No. 4,855,231 (*Pichia pastoris* and expression vectors therefor). Also, any species of *Candida* can be used. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the heterologous DNA sequences, sequences for  
25 polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschemper et al., Gene 10, 157 (1980)). This plasmid contains the *trp1* gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics 85, 12 (1977)). The presence of the  
30 *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include, but are not limited to, the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem.

255, 2073 (1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and Holland et al., Biochemistry 17, 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization.

In constructing suitable expression plasmids, the termination sequences associated with these genes may also be ligated into the expression vector 3' of the heterologous coding sequences to provide polyadenylation and termination of the mRNA.

A "reporter gene" is a gene that is operably linked to control sequences for expression of a gene of interest, and that encodes a gene product that can be detected for the purpose of evaluating expression of the associated gene of interest.

The "active state" of a receptor is the state at which the ligand that stimulates the receptor can bind to activate a signaling pathway controlled by the receptor.

An "EDG receptor agonist" is defined herein as a composition that is capable of combining with the active state of an EDG receptor to up-regulate a signaling pathway controlled by the receptor. An "EDG antagonist" is defined herein as a composition that is capable of combining with the EDG receptor in either the active or inactive state, thereby impeding the biological action of the receptor. An "inverse agonist" of EDG is defined herein as a composition that is capable of combining with the inactive state of an EDG receptor to down-regulate a signaling pathway controlled by the receptor.

A composition that "modulates" the activity of an EDG receptor is defined herein as a composition that is capable of evoking a change in the functional response mediated by said receptor.

As used herein, the terms "purified" or "isolated" are intended to refer to a molecule used in the present invention in an enriched or pure form obtainable from a natural source or by means of genetic engineering or synthetic chemistry. The purified protein, DNA or RNA of the invention may be useful in ways that the protein, DNA and RNA as they naturally

occur are not, such as identification of compounds selectively modulating the expression or the activity of the EDG-2 of the invention.

5 The isolated polypeptide and polypeptide fragments of an EDG means the EDG which is free of one or more components of its natural environment. Purified EDG includes purified EDG in recombinant cell culture. The enriched form of the receptor refers to a preparation containing said receptor in a concentration higher than natural, or in a cell where it is not found under native conditions e.g., a cellular membrane fraction comprising said receptor. If the receptor is in a pure form it is substantially free from other macromolecules, particularly from naturally occurring proteinaceous contamination. If desired, the receptor may be solubilized. Preferably, the receptor of the invention is in an active state meaning that it has both ligand binding and signal transduction activity. Receptor activity is measured according to methods known in the art, e.g., using a binding assay or a functional assay, e.g., an assay as described below.

10 The invention is further intended to encompass variants of the receptor of the invention. For example, a variant of an EDG receptor of the invention is a functional equivalent of said receptor. A functional equivalent is a protein displaying a physiological profile essentially identical to the profile characteristic of the particular member of the EDG family of receptors having the amino acid sequence set forth in SEQ ID NOS:1, 4, 5, 6, 7 or 8. The physiological profile *in vitro* and *in vivo* includes receptor effector function, electrophysiological and pharmacological properties, e.g., selective interaction with agonists or antagonists. Exemplary functional equivalents may be amino acid mutants including those having amino acid deletions, substitutions or insertions, and glycosylation variants. Functional equivalents may also include EDG receptors from a different organism. The present invention also encompasses methods for comparing the agonist profile of other EDG related receptors such as EDG-1 (Lee, M.-J., et al. (1996) *J. Biol. Chem.* **271**(19), 11272-11279; Hla, T., and Maciag, T. (1990) *J. Biol. Chem.* **265**(16), 9308-9313), EDG-3 (Yamaguchi, F., et al. (1996) *Biochem. Biophys. Res. Comm.* **227**, 608-614) and H218 (Okazaki, H., et al. (1993) *Biochem. Biophys. Res. Com.* **190**, 1104-1109; MacLennan, A. J., et al. (1994) *Mol. Cell. Neurosci.* **5**, 201-209) as well as the *Xenopus* high-affinity LPA receptor, PSP-24 (Guo, Z., et al. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14367-14372).

30 Covalent derivatives include, for example, aliphatic esters or amides of a receptor carboxyl group, O-acyl derivatives of hydroxyl group containing residues and N-acyl derivative of amino group containing residues. Such derivatives can be prepared by linkage



of functionalities to reactable groups which are found in the side chains and at the N- and C-terminus of the receptor protein. Polypeptides of this invention may be modified post-translationally (e.g., acetylation or phosphorylation).

The invention also encompasses methods wherein an EDG receptor is conjugated to a label capable of producing a detectable signal or other functional moieties. Suitable labels include, but are not limited to, radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent dyes, chemiluminescent dyes, bioluminescent compounds and magnetic particles.

A protein for use in the invention is obtainable from a natural source, e.g., by isolation, by chemical synthesis or by recombinant techniques.

The invention further provides a method for expressing the EDG receptors in host cells. Suitable host cells producing the receptor of the invention are multiplied *in vitro* or *in vivo*. Preferably, the host cells are transformed (transfected) with a hybrid vector comprising an expression cassette comprising a promoter and an *edg* DNA sequence. Subsequent to expression of the *edg* gene under control of the promoter, the specific EDG protein may be recovered. Recovery comprises, e.g., isolating the host cells comprising the receptor, e.g., from the culture broth.

The term "functional" or "biologically active", when used herein as a modifier of an EDG, refers to a polypeptide that is able to produce one or more of the functional characteristics exhibited by that specific native EDG. In one embodiment, functional means capable of binding its specific EDG ligand. In another embodiment, functional means that a signal is transduced as a consequence of binding of a ligand.

Suitable host cells include eukaryotic cells, e.g., animal cells, plant cells and fungi, and prokaryotic cells, such as Gram-positive and Gram-negative bacteria, e.g., *Escheria coli*.

Isolated polynucleotides (or nucleic acids) encoding a polypeptide substantially identical to an EDG protein or portions thereof are designated *edg*. The term polynucleotide as used herein, may be DNA or RNA, either coding or noncoding strands, *edg* cDNA, genomic DNA and synthetic or semi-synthetic DNAs and RNAs.

The invention includes modifications to *edg* DNA such as deletions, substitutions and additions particularly in the non-coding regions of genomic DNA. Such changes are useful to facilitate cloning and modify gene expression in methods of the present invention.

Various substitutions can be made within the coding region that either do not alter the amino acid residues encoded or result in conservatively substituted amino acid residues. Nucleotide substitutions that do not alter the amino acid residues encoded are useful for

optimizing gene expression in different systems. Suitable substitutions are known to those of skill in the art and are made, for instance, to reflect preferred codon usage in the particular expression systems.

5 The invention encompasses methods using functionally equivalent variants and derivatives of a particular *edg* which may enhance, decrease or not significantly affect the properties of the resultant EDG. For instance, changes in the DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect its properties, such as its ability to bind to LPA or analogs thereof.

10 An EDG is preferably incorporated into a vector (a virus, phage, or plasmid) which can be introduced by transfection or infection into a cell. The vector preferably includes one or more expression control sequences, in which case the cell transfected by the vector is capable of expressing the polypeptide. By "isolated DNA" is meant a single- or double-stranded DNA that is free of the genes that, in the naturally occurring genome of the animal from which the isolated DNA is derived, flank the *edg* gene. The term therefore includes, for example, either or both strands of an *edg* cDNA or an allelic variant thereof; a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryotic or eukaryotic cell; or a genomic DNA fragment (e.g., produced by PCR or restriction endonuclease treatment of human or other genomic DNA). The term also includes a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

15 Stringent conditions for both DNA/DNA and DNA/RNA hybridization assays are as described by Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, herein incorporated by reference. For example, see page 7.52 of Sambrook et al.

20 Given the guidance of the present invention, the nucleic acids used in the invention are obtainable according to the methods well known in the art.

30 For example, a DNA used in the invention is obtainable by chemical synthesis, by recombinant DNA technology or by PCR. Preparation by recombinant DNA technology may involve screening a suitable cDNA or genomic library. A suitable method for preparing a DNA or of the invention may, e.g., comprise the synthesis of a number of oligonucleotides, their amplification by PCR methods, and their splicing to give the desired DNA sequence.

Suitable libraries are commercially available or can be prepared from individual tissues or cell lines.

For an individual receptor related to an EDG, the expression pattern in different tissues may vary. Thus, in order to isolate cDNA encoding a particular EDG-related receptor, it is advantageous to screen libraries prepared from different suitable tissues or cells. As a screening probe, there may be employed a DNA or RNA comprising substantially the entire coding region of the *edg* or a suitable oligonucleotide probe based on said DNA. A suitable oligonucleotide probe (for screening involving hybridization) is a single stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or complementary to) any 14 or more contiguous bases set forth in SEQ ID NO:1, 4, 5, 6, 7 or 8, and preferably at least 18 contiguous bases that are the same as any 18 or more contiguous bases set forth in SEQ ID NO:1, 4, 5, 6, 7 or 8. The probe may be labeled with a suitable chemical moiety for ready detection. The nucleic acid sequences selected as probes should be of sufficient length and be sufficiently unambiguous so that false positive results are minimized.

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode ligand binding sites, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labeled with suitable label means for ready detection upon hybridization. For example, a suitable label means is a radiolabel. The preferred method of labeling a DNA fragment is by incorporating  $^{32}\text{P}$ -labeled  $\alpha$ -dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labeled with  $^{32}\text{P}$ -labeled  $\gamma$ -ATP and polynucleotide kinase. However, other methods (e.g., non-radioactive) may also be used to label the fragment or oligonucleotide, including, e.g., enzyme labeling and biotinylation.

After screening the library, e.g., with a portion of DNA including substantially the entire *edg* gene or a suitable oligonucleotide based on a portion of said DNA, positive clones are identified by detecting a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, e.g., by comparison with the sequences set forth herein, to ascertain whether they include a full length *edg* gene (i.e., if they include translation initiation and termination codons). If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If the library is genomic, then the overlapping clones may include exons

and introns. If the library is a cDNA library, then the overlapping clones will include an open reading frame. In both instances, complete clones can be identified by comparison with the DNAs and deduced amino acid sequences provided herein.

5 It is envisaged that the nucleic acid of the invention can be readily modified by nucleotide substitution, nucleotide deletion, nucleotide insertion or inversion of a nucleotide stretch, and any combination thereof. Such modified sequences can be used to produce a mutant EDG that differs from the receptors found in nature. Mutagenesis may be predetermined (site-specific) or random. A mutation that is not a silent mutation should not place sequences out of reading frames and preferably will not create complementary regions  
10 that could hybridize to produce secondary mRNA structures such as loops or hairpins.

The *edg* cDNA or genomic DNA can be incorporated into vectors for transfection of a host cell. Furthermore, the invention concerns a recombinant DNA which is a hybrid vector comprising at least one of the above mentioned genes.

15 The hybrid vectors of the invention comprise an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction endonuclease sites.

Preferably, the hybrid vector of the invention comprises an above described nucleic acid insert operably linked to an expression control sequence, in particular those described hereinafter.

20 Vectors typically perform two functions in collaboration with compatible host cells. One function is to facilitate the cloning of the *edg* gene, i.e., to produce useable quantities of the nucleic acid (cloning vectors). The other function is to provide for replication and expression of the gene constructs in a suitable host, either by maintenance as an extrachromosomal element or by integration into the host chromosome (expression vectors).  
25 A cloning vector comprises the DNAs as described above, an origin of replication or an autonomously replicating sequence, selectable marker sequences, and optionally, signal sequences and additional restriction sites. An expression vector additionally comprises expression control sequences essential for the transcription and translation of the *edg* gene. Thus, an expression vector refers to a recombinant DNA construct, such as a plasmid, a  
30 phage, recombinant virus or other vector that, upon introduction into a suitable host cell, results in expression of the cloned DNA. Suitable expression vectors are well known in the art and include those that are replicable in eukaryotic and/or prokaryotic cells.

Most expression vectors are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be amplified by insertion into the host genome. However, the recovery of the genomic *edg* gene is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise the gene. DNA can be amplified by PCR and directly transfected into the host cells without any replication component.

Advantageously, expression and cloning vectors contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the *edg* gene, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transfectants are placed under selection pressure in which only those transfectants that are uniquely adapted to survive are those which have taken up and are expressing the marker.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the *edg* gene. Suitable promoters may be inducible or constitutive. The promoters are operably linked to the *edg* gene by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native *edg* promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of an *edg*. However, heterologous promoters are preferred, because they generally allow for greater transcription and higher yields of EDG as compared to native *edg* promoter.

Promoters suitable for use with prokaryotic hosts include, for example, the  $\beta$ -lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (*trp*) promoter system and hybrid promoters such as the *tac* promoter. Their nucleotide sequences have been published thereby enabling the skilled worker to ligate them to the *edg* gene using linkers or

adaptors to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Dalgarno sequence operably linked to the *edg* gene.

5 The various DNA segments of the vector DNA are operably linked, *i.e.*, they are contiguous and placed in a functional relationship to each other. Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a manner known in the art. Suitable methods for constructing expression vectors, preparing *in vitro* transcripts, introducing DNA into host cells, and performing analyses for assessing *edg* expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), *in situ* hybridization, using an appropriately labeled probe based on a sequence provided herein, binding assays, immunodetection and functional assays.

15 Suitable methods for manipulation of polynucleotides include those described in a variety of references, including, but not limited to, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vol. 1-3, eds. Sambrook et al. Cold Spring Harbor Laboratory Press (1989); and *Current Protocols in Molecular Biology*, eds. Ausubel et al., Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates. Those skilled in the art will readily envisage how these methods may be modified, if desired.

20 The invention further provides host cells capable of producing *edg* and heterologous (foreign) polynucleotides encoding said receptor. The nucleic acids of the invention can be expressed in a wide variety of host cells, *e.g.*, those mentioned above, that are transformed or transfected with an appropriate expression vector. EDG proteins (or a portion thereof) may also be expressed as fusion proteins. Recombinant cells can then be cultured under conditions whereby the protein(s) encoded by the particular *edg* is (are) expressed.

25 Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, such as *E. coli*, *e.g.*, *E. coli* K-12 strains, DH5 $\alpha$  and HB101, or bacilli. Further host cells suitable for *edg*-encoding vectors include eukaryotic microbes such as filamentous fungi or in a preferred embodiment, yeast, *e.g.*, *Saccharomyces cerevisiae*.

30 The advantage of a yeast system is that yeast contain few G-protein coupled receptors and it is therefor a simple task to show that the response of the EDG receptor to a particular

phospholipid is dependent on the expression of the receptor since it is expressed from a galactose inducible promoter. This is in contrast to mammalian cells in which identity and distribution of LPA and other glycerophospholipids receptors is unclear. The results show that EDG-2 specifically responds to LPA. EDG-2 does not respond to other  
5 lysophospholipids or to diacyl-glycerophospholipids, in particular phosphatidic acid (PA) or to the related lipid messenger sphingosine-1-phosphate (SPP).

Higher eukaryotic cells include insect, amphibian and vertebrate cells, or mammalian cells. The methods for expressing proteins of interest in Sf9 cells are known in the art and are described in, for example *Current Protocols in Molecular Biology*, eds. Ausubel et al.,  
10 Greene Publishing and Wiley-Interscience: New York (1987) and references therein. In recent years, propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. The host cells referred to in this application comprise cells in *in vitro* culture as well as cells that are within a host animal.

Host cells are transfected or transformed with the above-captioned expression or  
15 cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation technique, by electroporation or by lipofectin-mediated  
20 transfection. Numerous methods of transfection are known to the skilled worker in the field. Successful transfection is generally recognized when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

Incorporation of cloned DNA into a suitable expression vector, transfection of  
25 eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press).

Transfected or transformed cells are cultured using media and culturing methods  
30 known in the art, preferably under conditions whereby the particular *edg* is expressed. Suitable culturing media are either commercially available or readily prepared.

The *edg* gene is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express the *edg* gene. The resulting cell line can then be

produced in amounts sufficient for reproducible qualitative and quantitative analysis of the effects of a receptor-specific agonist, antagonist or allosteric modulator. The transfected cells can then be employed in an drug screening assay provided hereinafter. Such drugs are useful in diseases associated with pathogenesis of LPA. Particularly useful for assessing the specific interaction of compounds with an EDG receptor are stably transfected cell lines expressing the EDG.

Cells expressing EDG polypeptides are useful for identifying substances that bind to a specific EDG. Identification of substances that bind to a specific EDG may be achieved by assessing the ability of a test compound to inhibit the binding of labeled ligand or analog thereof. Another method for identification of such substances involves assessing the ability of a test compound to inhibit specific antibody binding to an EDG.

Cells expressing EDG polypeptides are also useful for elucidating the signal transduction pathways to which EDG is coupled. By "signal transduction pathway" is meant the sequence of events that involves the transmission of a message from a cell-surface receptor to the cytoplasm. The signal will ultimately cause the cell to perform a particular function.

Thus, host cells expressing an EDG are also useful for drug screening and it is an object of the present invention to provide a method for identifying a compound or signal which modulates the activity of the EDG. The method includes exposing cells containing heterologous *edg*, wherein said cells produce functional EDG, to at least one compound or signal whose ability to modulate the activity of said EDG is sought to be determined. The cells are then monitored for changes caused by the modulation. Such an assay enables the identification of agonists, antagonists and allosteric modulators of EDG.

In a further aspect, the invention relates to an assay for identifying compounds that modulate the activity of any of the EDG family of receptors. The assay comprises the steps of:

- contacting cells expressing an active EDG and containing heterologous *edg* with at least one compound to be tested for its ability to modulate the activity of said receptor, and
- analyzing cells for a difference in second messenger level or receptor activity.

Additionally, to determine the specificity of the compound for a particular member of the EDG family of receptors, the assay can further comprise the steps of:

- contacting cells expressing one of the other EDG receptors, or the PSP-24 receptor and containing heterologous *edg* with the same compound tested above, and



- analyzing cells for a difference in second messenger level or receptor activity.

If the compound only effects receptor activity in one EDG family member, it is more specific, and may be preferable for certain therapeutic purposes, for example, to reduce the likelihood of undesired biological effects.

5

In particular, the invention covers an assay for identifying compounds that modulate the activity of EDG's, said assay comprising:

- contacting cells expressing an active EDG and containing heterologous *edg* with at least one compound to be tested for its ability to modulate the activity of said receptor, and
- monitoring said cells for a resulting change in second messenger activity.

10

The results obtained in these assays are compared to an assay suitable as a negative control.

Assay methods generally require comparison to various controls. A change in receptor activity or in second messenger level is "induced" by a test compound if such an effect does not occur in the absence of the test compound. An effect of a test compound on the receptor of the invention is "mediated" by the receptor if this effect is not observed in cells that do not express the receptor or express decreased amounts of the receptor.

15

As used herein, a compound or signal that modulates the activity of an EDG receptor refers to a compound or signal that alters the response pathway mediated by the EDG within a cell (as compared to the absence or decreased amount of said EDG). A response pathway is activated by an extracellular stimulus, resulting in a change in second messenger concentration or enzyme activity, or resulting in a change of the activity of a membrane-bound protein, such as a receptor or ion channel. A variety of response pathways can be utilized, including but not limited to, the adenylate cyclase response pathway, the phospholipase C/intracellular calcium ion response pathway or a response pathway involving activation of Ras or Rho.

20

25

Apoptosis represents another important response pathway that may be modulated by EDG agonists or antagonists. Suitable indications for therapeutic use of EDG agonists or antagonists that result in modulation of apoptotic pathways include, but are not limited to, ischemic heart disease, tumors, viral diseases such as HIV infection, neurodegenerative disorders, inflammatory bowel disease, hair loss, and rejection of organ transplants.

30

Thus EDG expressing cells may be employed for the identification of compounds, particularly low molecular weight molecules capable of acting as LPA agonists or

antagonists. Within the context of the present invention, an agonist refers to a molecule that is capable of interacting with one or more EDG's, thus mimicking the action of LPA. In particular, an LPA agonist is characterized by its ability to interact with EDG-2, thereby increasing or decreasing the stimulation of a response pathway within a cell. For example, an  
5 agonist increases or decreases a measurable parameter within the host cell, such as the concentration of a second messenger or modulation of apoptosis.

By contrast, in situations where it is desirable to decrease the activity of an EDG, antagonists are useful. Liliom et al. (1996) *Molec. Pharmacol.* 50:616-623; Bittman et al. (1996) *J. Lipid Res.* 37:391-398. Within the context of the present invention, an antagonist  
10 refers to a molecule that is capable of interacting with an EDG, but which does not stimulate a response pathway within a cell. In particular, LPA antagonists are generally identified by their ability to interact with EDG-2, and thereby reduce the ability of the natural ligand to stimulate a response pathway within a cell, e.g., by interfering with the binding of LPA to EDG-2 or by inhibiting other cellular functions required for the activity of EDG-2. For  
15 example, in a suitable assay, e.g., an assay involving suitable eukaryotic cells expressing EDG-2, a LPA antagonist is capable of modulating the activity of EDG-2 such that the ability of the natural ligand to activate the map kinase pathway is reduced. Yet another alternative to achieve an antagonistic effect is to rely on overexpression of antisense *edg-2* RNA. Preferred is an agonist or antagonist selectively acting on EDG-2.

20 An allosteric modulator of an EDG interacts with the receptor protein at another site than that recognized by any one of its particular ligands, thus acting as agonist or antagonist. Therefore, the screening assays described herein are also useful for detecting an allosteric modulator of a receptor of the invention. For example, an allosteric modulator acting as agonist may enhance the specific interaction between EDG-2 and LPA. For instance, if an  
25 allosteric modulator acts as an antagonist, it may interact with the receptor protein in such a way that binding of the agonist is functionally less effective. Examples include local anesthetics such as procaine, lidocaine, dibucaine and tetracaine.

An *in vitro* assay for a LPA agonist or antagonist may require that an EDG is produced in sufficient amounts in a functional form using recombinant DNA methods. An  
30 assay is then designed to measure a functional property of the EDG, e.g., interaction with LPA. Production of EDG is regarded as occurring in sufficient amounts if activity of the receptor results in a measurable response.

For example, mammalian cells (available, e.g., from the American Tissue Type Culture Collection) are grown in appropriate culture medium. An EDG expression plasmid is transiently transfected into the cells, e.g., by calcium-phosphate precipitation. Ausubel, F.M. et al. (1993). Cell lines stably expressing the EDG may be generated, e.g., by lipofectin-mediated transfection with EDG expression plasmids and a plasmid comprising a selectable marker gene. Southern and Berg (1982) *J. Mol. Appl. Genet.* 1:327-341. Cells surviving the selection are isolated and grown in the selection medium. Resistant clonal cell lines are analyzed, e.g., for immunoreactivity with EDG-specific antibodies or by assays for EDG functional responses following agonist addition. Cells producing EDG are used in a method for detecting compounds binding to EDG or in a method for identifying an EDG ligand agonist or antagonist.

Compound bound to the target EDG may modulate functional properties of EDG and may thereby be identified as an EDG ligand agonist or antagonist in a functional assay. Functional assays are used to detect a change in the functional activity of EDG's, for instance, as a result of the interaction of the compound to be tested with an EDG. A functional response is a change (difference) in the concentration of a relevant second messenger influenced by the receptor of the invention within cells expressing a functional EDG (as compared to a negative control). Those of skill in the art can readily identify an assay suitable for detecting a change in the level of an intracellular second messenger indicative of the expression of active EDG-2 (functional assay). Examples include cAMP assays (see, e.g., Nakajima et al. (1992) *J. Biol. Chem.* 247:2437-2442); Tigyi et al. (1996) *J. Neurochem.* 66:549-558) measuring changes in inositol 1,4,5-triphosphate levels (Tigyi et al. (1996) *J. Neurochem.* 66:537-548), measuring  $\text{Cl}^-$  ion efflux (Postma et al. (1996) *EMBO J.* 15:63-72; Watsky (1995) *Am. J. Physiol.* 269:C1385-C1393), or measuring changes in intracellular  $\text{Ca}^{2+}$  levels (Tigyi et al. (1996) *J. Neurochem.* 66:537-548).

More specifically, according to the invention, a method for detecting a LPA agonist comprises the steps of (a) exposing a compound to EDG-2 coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with EDG-2 and an associated response through the pathway, and (b) detecting an increase or decrease in the stimulation of the response pathway resulting from the interaction of the compound with EDG-2, relative to the absence of the tested compound and therefrom determining the presence of a LPA agonist.

A method for identifying a LPA antagonist comprises the steps of (a) exposing a compound in the presence of a known LPA agonist to EDG-2 coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the agonist with the receptor and an associated response through the pathway, and (b) detecting an inhibition of the stimulation of the response pathway induced by the agonist, said inhibition resulting from the interaction of the compound with EDG-2, relative to the stimulation of the response pathway by the LPA agonist alone and determining therefrom the presence of a LPA antagonist. Inhibition may be detected if the test compound competes with the LPA agonist for EDG-2. Compounds which may be screened utilizing such a method include, but are not limited to, blocking antibodies specifically binding to EDG-2. Furthermore, such an assay is useful for the screening for compounds interacting with LPA. In this case, the agonistic effect is neutralized or reduced, e.g., by binding of the test compound to the agonist, thus affecting agonist interaction with the receptor. Examples are soluble EDG-2 fragments comprising part or all of the ligand binding domain.

Preferentially, interaction of an agonist or antagonist with EDG-2 denotes binding of the agonist or antagonist to said EDG-2.

As employed herein, conditions and times sufficient for interaction of an LPA agonist or antagonist candidate with the receptor will vary with the source of the receptor, however, conditions generally suitable for binding occur between about 4°C and about 40°C, preferably between about 4°C and about 37°C, in a buffer solution between 0 and 2 M NaCl, preferably between 0 and 0.9 M NaCl, with 0.1 M NaCl being particularly preferred, and within a pH range of between 5 and 9, preferably between 6.5 and 8. Sufficient time for the binding and response will generally be between about 1 ms and about 24 h after exposure.

Within one embodiment of the present invention, the response pathway is a membrane-bound Map Kinase pathway, and, for an agonist, the step of detecting comprises measuring a reduction or increase, preferably a reduction, in *lacZ* production by the membrane-bound response pathway, relative to the *lacZ* production in the relevant control setup. For the purpose of the present invention, it is preferred that the reduction or increase in *lacZ* production be equivalent or greater than the reduction or increase induced by LPA applied at a concentration corresponding to its  $IC_{50}$  value. For an antagonist, the step of detecting comprises measuring in the presence of the antagonist a smaller LPA-induced decrease or increase in *lacZ* production by the membrane-bound response pathway, as compared to the *lacZ* production in the absence of the antagonist. The measurement of *lacZ*

may be performed after cell destruction or by a *lacZ* sensitive molecular probe loaded into the cell.

Yeast contains multiple MAP kinase cascades that are functionally analogous to the Mitogen Activated Protein Kinase (MAPK) cascade in mammalian systems (Brewster, J. L., et al. (1993) *Science* **259**, 1760-1763; Irie, K., et al. (1993) *Mol. Cell. Biol.* **13**, 3076-3083; Neiman, A. M., and Herskowitz, I. (1993) *Trends Genet.* **9**, 390-394; Chang, E. C., et al. (1994) *Cell* **79**, 131-141). A schematic of the *S. cerevisiae* pheromone response pathway and the relevant genetic components are shown in Figure 1. The parental yeast strain, SY2069, contains the *FUS1* promoter fused to *lacZ* and *HIS3* integrated into different chromosomal loci and carries the *far1*-bad allele. The *FAR1* gene product is required for cell cycle arrest following exposure to mating pheromone (see Figure 1). By deleting this gene, the cells are able to grow in the presence of MAP kinase activation. In addition, a null mutation in the *SST2* gene was created because it has been previously reported that the Sommatostatin receptor can efficiently couple to the endogenous yeast heterotrimeric G-protein after mutationally inactivating the *SST2* gene (Price, L. A., Kajkowski, E. M., Hadock, J. R., Ozenberger, B. A., and Pausch, M. H. (1995) *Mol. Cell. Biol.* **15**(11), 6188-6195). *SST2* encodes a GTPase activating protein (GAP) for the *GPA1* gene product, the  $G\alpha$  subunit required for mating pheromone signal transduction. The effect of inactivating *SST2* is that *Gpa1* remains in the GTP-bound state longer and thus permits signaling through the  $\beta\gamma$  dimer to proceed at a higher rate, resulting in a higher signal from the receptor.

Further cell-based screening assays can be designed, e.g., by constructing cell lines in which the expression of a reporter protein, i.e., an easily assayable protein, such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase (CAT) or luciferase, is dependent on the function of an EDG. The resulting DNA construct comprising the enzyme DNA is stably transfected into a host cell. The host cell is then transfected with a second DNA construct containing the *edg* gene operably linked to additional DNA segments necessary for the expression of the receptor.

Also encompassed by methods of the present invention is the use of the EDG family of receptors in an expression vector for radioligand binding assays, such as that described in Price, L. A., et al. (1996) *Mol. Pharmacol.* **50**(4), 829-837. Such methods can be used for detecting compounds that compete with known EDG binding ligands for binding to the EDG related receptors. For example, first labeled 18:1 lysophosphatidic acid ligand is introduced to a sample containing yeast cells expressing EDG-2 receptor to allow binding of the 18:1

LPA to the EDG-2 receptor. Next, a composition to be tested for its ability to modulate the interaction of 18:1 LPA and its receptor is introduced to the yeast cell. Finally, the yeast sample is evaluated for an increase or decrease in the amount of labeled agonist bound to the yeast cell or membranes purified from the yeast cell. Methods of labeling compounds, and labels themselves, are known in the art and include, but are not limited to, radioisotopes, enzymes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds, substrate cofactors and inhibitors. See, for examples of patents teaching the use of such labels, U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. By these methods, one can identify the presence of an EDG related receptor modulator, such as an antagonist, inverse agonist or agonist.

The *edg* gene family is useful for a variety of studies. These include, but are not limited to, testing LPA analogs for agonistic/antagonistic activity and for EDG-2 specificity when compared to other EDG family members; dissecting the molecular signal transduction mechanism, analyzing receptor-ligand interactions by site-directed mutagenesis; determining the levels and distribution of the receptors; cloning related receptors; and determining the mechanism of tissue-specific responses to LPA and other EDG family ligands.

Recently, it has been discovered that a lysophospholipid effects *Candida* adherence in vitro. Prakobphol, A., et al. "Plamitoyl carnitine, a lysophospholipase-transacylase inhibitor, prevents *Candida* adherence in vitro," FEMS Microbiol Lett (1997) 151:89-94. By identifying a lysophosphatidic acid receptor in *Candida*, and inserting that receptor into an assay of the present invention, one can screen for potential anti-fungal agents that modulate the interaction of LPA and such a receptor.

Methods of the present invention can be used to identify an LPA receptor from a heterologous fungal species, such as *Candida*. For example, one can a) contact recombinant host cells, modified to contain a fragment of at least 18 consecutive nucleotides of DNA selected from the genomic library of a chosen fungal species, which is operably linked to control sequences for expression, with LPA. Next, one can b) analyze the cells for a change in functional response to said LPA. Finally, one can repeat steps a) and b) with different fragments from the genomic library of the heterologous fungus until a fragment is identified that evokes a change in functional response upon treatment with LPA.

The following examples are provided to illustrate but not limit the invention.

## EXPERIMENTAL

The following abbreviations, used in the experimental section and throughout the specification, are herein set forth: LPA (lysophosphatidic acid), LPE (lysophosphatidylethanolamine), LPS (lysophosphatidylserine), LPC (lysophosphatidylcholine), LPG (lysophosphatidylglycerol), PA (phosphatidic acid), PE (phosphatidylethanolamine), PS (phosphatidylserine), PC (phosphatidylcholine), PG (phosphatidylglycerol), Sph-1-P (shingosine-1-phosphate), BBS (Bicarbonate buffered saline).

### EXAMPLE 1

#### Construction of the EDG-1, EDG-2, EDG-3, EDG-4 and EDG-5 Expression Plasmids and Expression in Yeast

SY2069 (*Mata, far1-bad3, HIS3::pFUS1::HIS3, mfa2-Δ1::pFUS1::lacZ, ura3, leu2, adel, arg4, trp1*) was used to derive an *sst2*<sup>-</sup> strain for subsequent studies. *SST2* was disrupted using pBC14 (Dohlman, H. G., et al. (1996) *Mol. Cell. Biol.* **16**(9), 5194-5209). pBC14 was digested with NcoI and transformed into SY2069 by lithium acetate using the *URA3* gene for selection. *Ura*<sup>+</sup> colonies were grown in non-selective media (YEPD) and plated onto media containing 5-Flouro-orotic Acid (5-FOA, Sigma). The resultant 5-FOA resistant isolates were tested for the supersensitive phenotype by assaying *lacZ* activity in response to  $\alpha$ -factor (data not shown). One *sst2*<sup>-</sup> strain was named JEY5 and used in all subsequent studies. Yeast were grown in SC + 2% Galactose or 2% Glucose media lacking Uracil.

Construction of EDG-2 Expression Plasmid and Expression in Yeast: The EDG-2 coding region was amplified by RT-PCR using Pfu DNA polymerase under conditions described by the supplier (Stratagene). The template for RT-PCR was cDNA (5ng) that was reverse transcribed from human fetal brain total RNA (Clontech) using SuperScript II Reverse Transcriptase as described by the supplier (Gibco BRL). 1 $\mu$ M each of the following primers, FP; 5'-GCGATAGGATCCATCATGGCTGCCATCTCTACTTC-3' (SEQ ID NO. 2) and RP; 5'-GCGATACTCGAGCTAAACCACAGAGTGATCATTGC-3' (SEQ ID NO. 3), were used for RT-PCR. Oligonucleotide Synthesis and DNA Sequencing: PCR primers and DNA sequencing primers were synthesized by the phosphoramidite method with an Applied Biosystems model 394 synthesizer, purified by polyacrylamide gel electrophoresis and desalted on Sep-Pak C<sub>18</sub> cartridges (Waters Associates, Milford, MA). The edg-2 cDNA

was sequenced in pYEura3 by the dideoxy chain termination method using the T7 Sequenase7-deaza-dGTP sequencing kit as described by the supplier (Amersham Life Science). The primers were designed based on the human *edg-2* cDNA sequence submitted to Genbank by Zondag and Moolenaar (accession no. Y09479) and included restriction site extensions for subcloning into the pYEura3 vector (Stratagene). This placed the cDNA under the control of a galactose-inducible promoter (UASgal). The resulting plasmid was used to transform JEY5 by the lithium acetate method.

Construction of EDG-1, EDG-3, EDG-4 and EDG-5 Expression Plasmid and Expression in Yeast: The other EDG Expression Plasmids were prepared by the same method described above for EDG-2, except that the coding regions were amplified by RT-PCR using the following primers, respectively:

EDG-1:FP; 5'-GCGCGGGATCCACCATGGGGCCCACCAGCGTCCCG-3' (SEQ ID NO.10)  
 RP; 5'-GCGCGGTCTGACGGAAGAAGAGTTGACGTTTCC-3' (SEQ ID NO. 11)

EDG-3:FP; 5'-GCGCGGGATCCACCATGGCAACTGCCCTCCCG-3' (SEQ ID NO. 12)  
 RP; 5'-GCGCGGTCTGACTCAGTTGCAGAAGATCCCATTTC-3' (SEQ ID NO. 13)

EDG-4:FP; 5'-ATCAGCGGATCCACCATGGTCATCATGGGCCAGT-3' (SEQ ID NO. 14)  
 RP; 5'-AGTTCACTCGAGTCAGTCCTGTTGGTTGGGTTG-3' (SEQ ID NO. 15)

EDG-5:FP; 5'-GCGCGGGATCCACCATGGGCGGTATATACTCAGAG-3' (SEQ ID NO. 16)  
 RP; 5'-GCGCGGTCTGACTCAGACCACTGTGTTGCCC-3' (SEQ ID NO. 17).

Expression of the EDG-2: To test the effects of the *SST2* gene product on the *edg-2* response to LPA, JEY5 (*sst2Δ*) expressing the EDG-2 receptor was compared to the parental SY2069 strain (*SST2*<sup>+</sup>). Figure 2 shows that the *SST2*<sup>+</sup> strain was unresponsive to LPA whereas the *sst2*<sup>-</sup> derivative was activated by 200μM LPA. As a control, JEY5+EDG-2 was assayed in 2% glucose such that the *GALI* promoter would be repressed and thus not expressing the *edg-2* gene (glucose repression is described in detail by Johnston, M., and Carlson, M. (1992) in *The molecular and cellular biology of the yeast Saccharomyces: gene expression* (Broach, J. R., Pringle, J. R., and Jones, E. W., eds), pp. 193-281, Cold Spring Harbor Laboratory Press, Plainview, N.Y.).



*lacZ* assays in response to phospholipids: JEY5 + pJE15 was grown on SC media containing either 2% galactose or 2% glucose lacking uracil to an approximate optical density of 0.1-0.5 prior to the addition of lipid or  $\alpha$ -factor. LPA and other glycerophospholipids (Avanti Polar Lipids) were dissolved in chloroform and dried down under vacuum immediately prior to experiments and resuspended in BBS/EDTA (50mM  $\text{NH}_4\text{HCO}_3$ , 104mM NaCl, 250 mM EDTA·2Na) at 1mg/ml with sonication until the solution was clear. Sphingosine-1-phosphate (Matreya) was resuspended in ethanol/water (9:1) pH 3.0 immediately prior to use. Fatty Acid Free Bovine Serum Albumin was obtained from Sigma (St. Louis, MO) and used at 0.1mg/ml in BBS/EDTA. Cells were allowed to grow for the indicated time (7 hours for dose response experiments) prior to assaying. 100 $\mu$ l of yeast culture were then added to 900 $\mu$ l assay buffer (per liter: 60 mM  $\text{Na}_2\text{HPO}_4$ , 40mM  $\text{NaH}_2\text{PO}_4$ , 10mM KCl, 0.1 mM  $\text{MgSO}_4$ , pH 7.0 plus 2.7ml  $\beta$ -mercaptoethanol per liter) plus 50 $\mu$ l 0.1%SDS + three drops Chloroform. Cells were vortexed for 10 seconds and incubated for 5 minutes at 28°C. 200 $\mu$ L of 4mg/ml o-nitrophenol- $\beta$ -D-galactopyranoside (ONPG, Sigma) were added and the reaction was incubated 30 minutes at 28°C. The assay was stopped by the addition of 500 $\mu$ l 1M  $\text{Na}_2\text{CO}_3$ . Color development was measured at  $A_{420}$  and normalized to  $A_{600}$ . Units were expressed as Miller Units.

Figure 2 shows that the induction of *lacZ* activity is dependent on 1) the pYEUra3-Edg2 plasmid being present in the yeast cell, 2) the yeast cell containing the pYEUra3-Edg2 plasmid being grown on the sugar galactose such that the UASgal promoter which drives the expression of the Edg2 gene is induced and 3) lysophosphatidic acid being present.

To further characterize the LPA response to EDG-2, the time and dose dependency of LPA activation was tested. As seen in figure 3A, LPA results in a time-dependent increase in *lacZ* activity as compared to vector control with a maximal four-fold stimulation of activity at 7 hours. The dose response of EDG-2 to LPA is shown in Figure 3B ( $\text{E.C.}_{50}$ =20 $\mu$ M-30 $\mu$ M). LPA concentrations above 600  $\mu$ M could not be tested due to toxicity. However, the dose response curve can be seen to plateau suggesting that maximal activity has been reached. This toxicity was seen in other glycerophospholipids tested at 200 $\mu$ M (see below). However, the response to LPA was significantly above the vector control suggesting that EDG-2 confers LPA responsiveness upon yeast. LPA resuspended in BBS/EDTA + fatty acid free albumin showed the same activity as did freely soluble LPA (data not shown and see below).

The results support that the expression of EDG-2 in yeast faithfully reconstitutes many of the key properties of an LPA receptor.

5

### EXAMPLE 2

EDG-2 responds selectively to LPA and not to other lysophospholipids or to corresponding diacyl-glycerophospholipids

Yeast does not have endogenous receptors for glycerphospholipids such as LPA. Therefore, yeast represented an excellent naïve system to evaluate the agonist binding specificity of EDG-2. Lysophosphatidylethanolamine (LPE), -serine (LPS), -glycerol (LPG) and -choline (LPC) and Sphingosine-1-phosphate (SPP) were tested over the same dose range as LPA.

The results are consistent with EDG-2 being a functional, specific LPA receptor. As seen in Figure 4, no other lyso-glycerolphospholipid or Sphingosine-1-phosphate activated EDG-2 as well as did LPA at concentrations up to 200  $\mu$ M, the highest concentration tested due to toxicity. The results of a similar experiment testing the effects of the diacyl-glycerophospholipids are seen in Figure 5. In this experiment, no diacyl-glycerophospholipid significantly activated EDG-2 except phosphatidic acid (PA). However, the activity of PA may be due to contaminating LPA as determined by HPLC (data not shown).

20

### EXAMPLE 3

Acyl-chain length dependency of EDG-2 activity by LPA

Due to the apparent specificity of EDG-2 for LPA, the dependency of the acyl-chain length of the LPA molecule on EDG-2 activation was also determined. Six forms of LPA were tested: 24:1, 18:1 (Oleoyl), 18:0 (Stearoyl), 16:0 (Palmitoyl), 14:0 (Myristoyl) and 6:0 (Caproyl). The experiment was carried out as described in Example 2, above, using the four forms of LPA.

Figure 6 shows that those LPA molecules containing 16 or 18 carbons activated EDG-2. This experiment was repeated with the LPA analogs suspended in 0.1 mg/ml fatty acid free BSA with similar results (data not shown).

30

### EXAMPLE 4

LPA in a liposomal formulation is effective in EDG-2 activation

To test the effects of presenting LPA to the yeast cells as a liposome rather than as freely soluble LPA or bound to albumin, liposomes were formed with either phosphatidylcholine or phosphatidylglycerol. To formulate the liposomes, LPA and dioleoyl-phosphatidylcholine or 1-palmitoyl-2-oleoyl-phosphatidylglycerol were mixed at a weight ratio of 1:9 (LPA:PC or PG) in Chloroform solution, dried under vacuum (Savant) and resuspended in BBS/EDTA to a total lipid concentration of 10mg/ml (LPA concentration = 1mg/ml). The resultant opaque suspension was sonicated until the solution was clear (approximately 10 minutes, Lab Supplies Co., Hicksville, NY). The liposome size ranged from 50nm to 80nm as determined on a Coulter N4 Plus Particle Sizer. (Coulter).

Figure 7 shows that LPA-containing liposomes showed equivalent activity towards EDG-2 as did freely soluble LPA. Phosphatidylcholine and phosphatidylglycerol as liposome preparations gave no activity on their own (Figure 4). These results support that the form in which LPA is presented does not significantly effect its ability to activate EDG-2; albumin-bound, freely soluble and liposomal formulations of LPA all activated EDG-2 with equal efficacy.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

We claim:

1. A method for identifying compounds that modulate the activity of an EDG related receptor, comprising the steps of:
  - a) contacting recombinant host cells, modified to contain a fragment of at least 18 consecutive nucleotides of DNA selected from the group comprising SEQ. ID. NOS. 1, 4, 5, 6, 7 and 8, which is operably linked to control sequences for expression, with at least one compound or signal whose ability to modulate the activity of the particular EDG receptor is sought to be determined, and
  - b) analyzing the cells for a difference in functional response mediated by said receptor.
2. A method according to claim 1, comprising:
  - a) contacting said cells with at least one compound or signal whose ability to modulate the activity of said receptor is sought to be determined, and
  - b) monitoring said cells for a change in the level of a particular second messenger.
3. A method for modulating the signal transduction activity of an EDG receptor, comprising contacting said receptor with an effective amount of at least one compound identified by the method of claim 1.
4. An agonist, antagonist or allosteric modulator identified by the method of claim 1.
5. A method for detecting an LPA agonist or an allosteric modulator of an LPA receptor having agonistic activity comprising the steps of:
  - a) exposing a compound to the EDG-2 receptor coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with the EDG-2 receptor and an associated response through the pathway, and
  - b) detecting an increase or a decrease in the stimulation of the response pathway resulting from the interaction of the compound with the EDG-2 receptor, relative to the

absence of the tested compound and therefrom determining the presence of an agonist or an allosteric modulator.

6. A method for detecting an LPA antagonist or an allosteric modulator of LPA receptor having antagonistic activity comprising the steps of

- a) exposing a compound to the EDG-2 receptor coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with the EDG-2 receptor and an associated response through the pathway, and
- b) detecting an increase or a decrease in the stimulation of the response pathway resulting from the interaction of the compound with the EDG-2 receptor, relative to the absence of the tested compound and therefrom determining the presence of an antagonist or an allosteric modulator.

7. A composition comprising a viable yeast cell transformed with a plasmid expressing an EDG related receptor protein selected from the group comprising SEQ. ID NO: 1, 4, 5, 6, 7 and 8, a pheromone responsive element and a reporter gene, wherein the reporter gene detects the activation of the LPA receptor.

8. The composition of claim 7, wherein said EDG related receptor protein has an amino acid sequence corresponding to SEQUENCE ID NO. 1.

9. The composition of claim 7, wherein said yeast cell is *Saccharomyces cerevisiae*.

10. The composition of claim 7, wherein said reporter gene is *lacZ*.

11. A method for identifying compounds which modulate the activity of EDG-2, comprising the steps of:

- a) contacting recombinant host cells, modified to contain the DNA of SEQ. ID. NO. 1 which is operably linked to control sequences for expression, with at least one compound or signal whose ability to modulate the activity of the EDG-2 LPA receptor is sought to be determined, and

- b) analyzing the cells for a difference in functional response mediated by said receptor.

12. A method for modulating the signal transduction activity of the EDG-2 LPA receptor, comprising contacting said receptor with an effective amount of at least one compound identified by the method of claim 11.

13. An agonist, antagonist, inverse agonist, or allosteric modulator identified by the method of claim 11.

14. A method for detecting an LPA agonist or an allosteric modulator of an LPA receptor having agonistic activity comprising the steps of:

- a) exposing a compound to the EDG-2 receptor (SEQ ID NO. 1) coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with the EDG-2 receptor and an associated response through the pathway, and
- b) detecting an increase or a decrease in the stimulation of the response pathway resulting from the interaction of the compound with the EDG-2 receptor, relative to the absence of the tested compound and therefrom determining the presence of an agonist or an allosteric modulator.

15. A method for detecting an LPA antagonist or an allosteric modulator of LPA receptor having antagonistic activity comprising the steps of

- a) exposing a compound to the EDG-2 receptor (SEQ ID NO. 1) coupled to a response pathway, under conditions and for a time sufficient to allow interaction of the compound with the EDG-2 receptor and an associated response through the pathway, and
- b) detecting an increase or a decrease in the stimulation of the response pathway resulting from the interaction of the compound with the EDG-2 receptor, relative to the absence of the tested compound and therefrom determining the presence of an antagonist or an allosteric modulator.

16. A method for detecting inverse agonists of LPA, comprising the steps of

- a) exposing a compound and LPA to the EDG-2 receptor (SEQ ID NO. 1) coupled to a response pathway, under conditions and for a time sufficient to allow interaction of LPA with the EDG-2 receptor and an associated response through the pathway; and

b) detecting a change in the stimulation of the response pathway, relative to the absence of the tested compound and therefrom determining the presence of an inverse agonist of LPA.

17. A genetically engineered viable yeast cell transformed with plasmids expressing an EDG related receptor protein having an amino acid sequence corresponding to Seq. ID. NO. 1, 4, 5, 6, 7 or 8, and a reporter gene, wherein the reporter gene detects the activation of the EDG related receptor upon the binding of modulators of the EDG related receptor.

18. The yeast cell of claim 17 wherein said yeast cell is selected from the group consisting of *Saccharomyces cerevisiae* and *Saccharomyces pombe*.

19. The yeast cell of claim 17, wherein the reporter gene is lac Z.

20. The yeast cell of claim 17, wherein said EDG related receptor protein has an amino acid sequence corresponding to Seq. ID. NO. 1.

21. A method for analyzing the specificity of compounds which modulate the activity of an EDG related receptor, comprising the steps of:

a) contacting a first group of recombinant host cells, modified to contain a first fragment of at least 18 consecutive nucleotides of DNA selected from the group comprising SEQ. ID. NOS. 1, 4, 5, 6, 7 and 8, which is operably linked to control sequences for expression, with at least one compound or signal whose ability to modulate the activity of the particular EDG receptor is sought to be determined,

b) contacting a second group of recombinant host cells, modified to contain a second fragment of at least 18 consecutive nucleotides of a different DNA selected from the group comprising SEQ. ID. NOS. 1, 4, 5, 6, 7 and 8, and

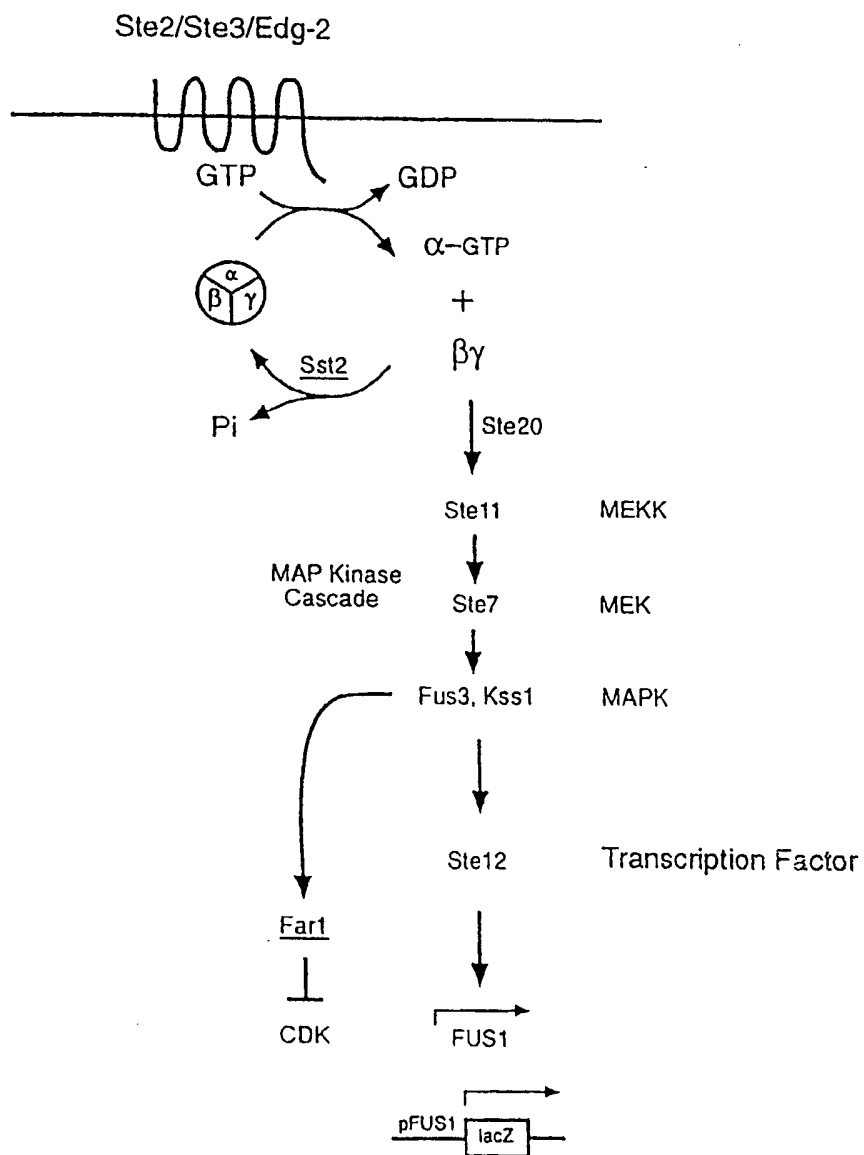
c) analyzing the cells from steps a) and b) for a difference in functional response mediated by said first and said second receptors.

22. A method according to claim 21, comprising:

- a) contacting said first and said second group of recombinant host cells with at least one compound or signal whose ability to modulate the activity of said receptor is sought to be determined, and
  - b) monitoring said first and said second group of recombinant host cells for a change in the level of a particular second messenger.
23. A method of detecting compounds that modulate the interaction between a ligand of an EDG related receptor and the EDG related receptor, comprising:
- a) exposing a labeled ligand of an EDG related receptor to a cell expressing said EDG related receptor;
  - b) exposing a labeled compound that is believed to interact with an EDG related receptor to said cell, and
  - c) detecting a change in the amount of labeled ligand bound to said cell.
24. A method of identifying an LPA receptor from a heterologous fungal species, comprising:
- a) contacting recombinant host cells, modified to contain a fragment of at least 18 consecutive nucleotides of DNA selected from the genomic library of said fungal species, which is operably linked to control sequences for expression, with LPA,
  - b) analyzing the cells for a change in functional response, and
  - c) repeating steps a) and b) with different fragments from said genomic library until a fragment is identified that evokes a change in functional response of said cells upon treatment with LPA.

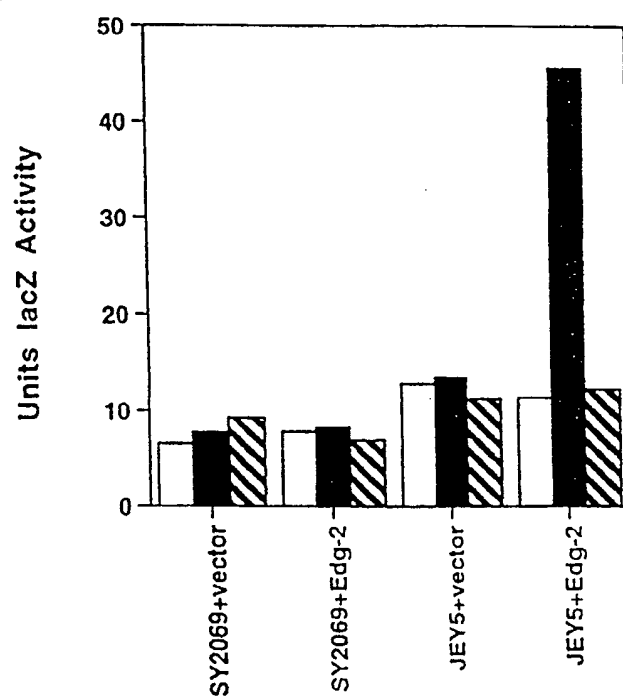


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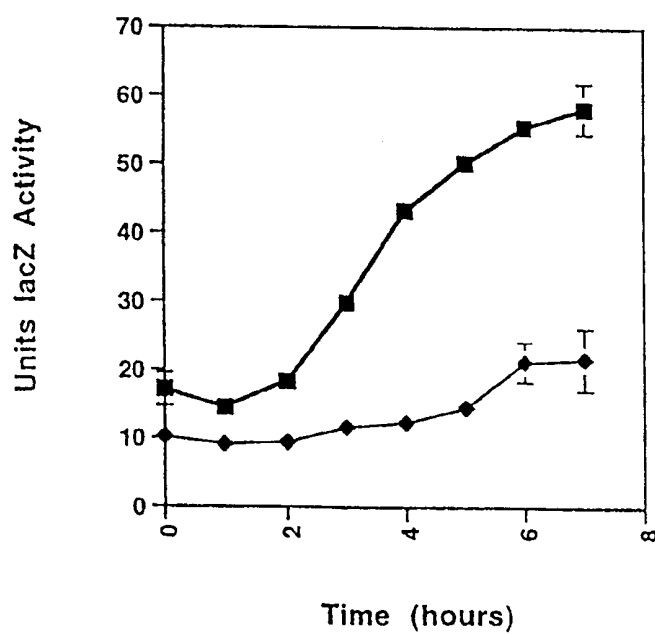
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Figure 2



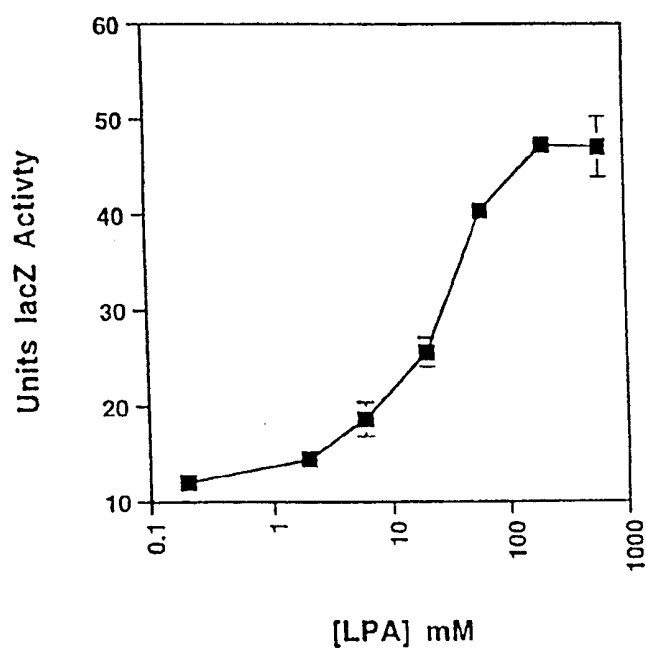
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Figure 3A



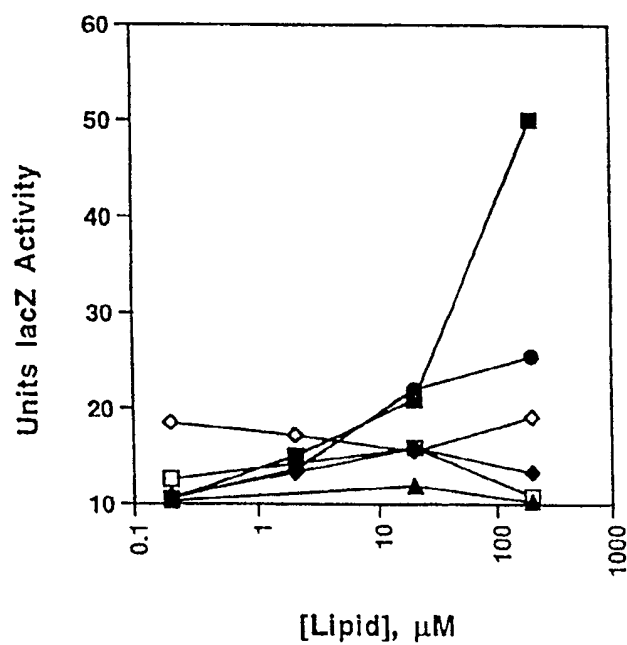
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Figure 3B



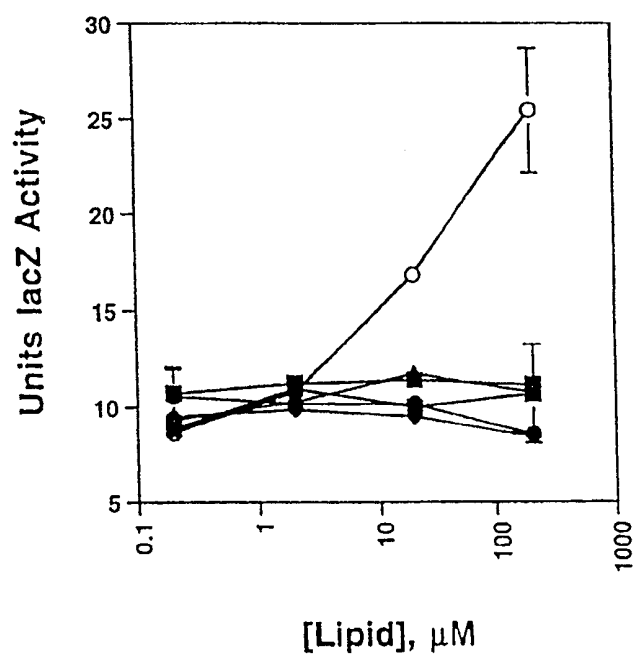
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Figure 4



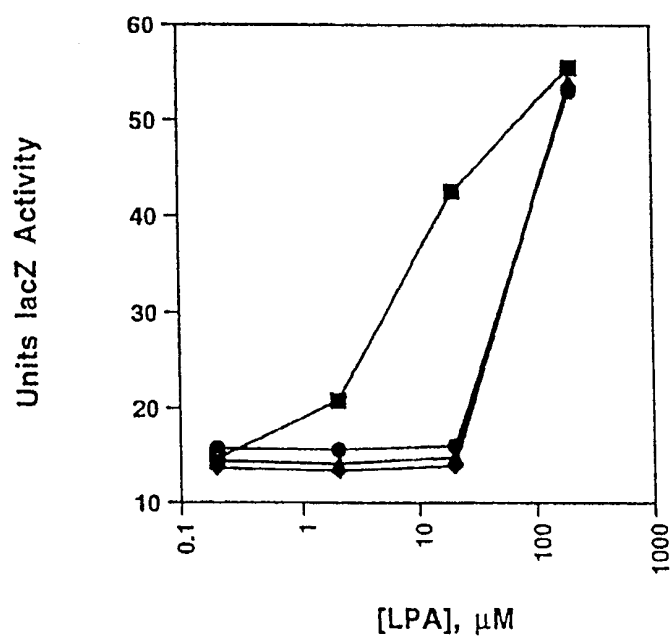
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Figure 5



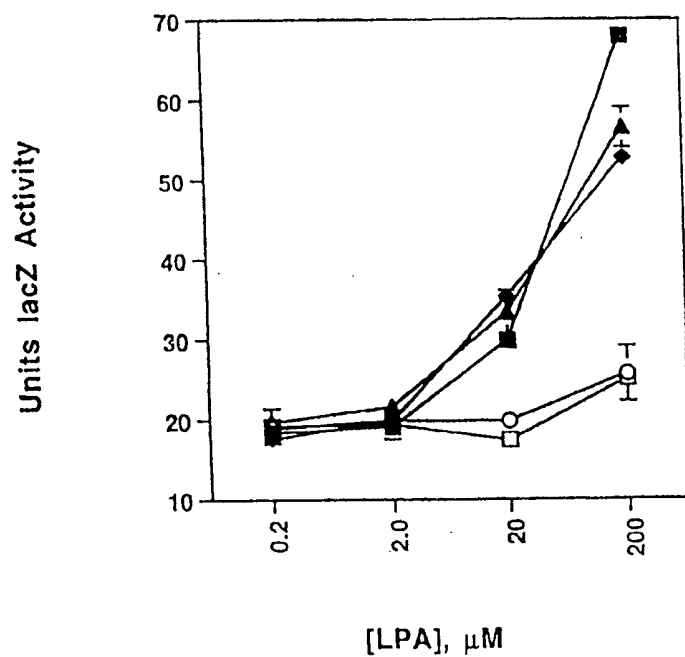
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Figure 6



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Figure 7





## SEQUENCE LISTING

<110> Erikson, James  
 Goddard, J. Graham  
 Kiefer, Michael  
 LXR Biotechnology, Inc.

<120> METHODS FOR DETECTING COMPOUNDS WHICH MODULATE THE  
 ACTIVITY OF AN LPA RECEPTOR

<130> 4147-6-PCT

<140> PCT/US98/21315

<141> 1998-10-09

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gccatgctac ccggtatgac tgggtaatga ggacagactg tggacacccc atctacctga 1570
gtctgattct ttagcagcag agactgaggg gtgcagagtg tgagctggga aaggtttgtg 1630
gtctccttga gcctccaggg actggcctgt cccaataga attgaagcag tccacgggga 1690
ggggatgata caaggagtaa acctttcttt aactcaaaaa aaaa 1734

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<210> 7  
 <211> 1059  
 <212> DNA  
 <213> Rattus sp.

<220>  
 <221> CDS  
 <222> (1)..(1056)



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Met Gly Gly Leu Tyr Ser Glu Tyr Leu Asn Pro Glu Lys Val Gln Glu	
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cac tac aat tac acc aag gag acg ctg gac atg cag gag acg ccc tcc	96
His Tyr Asn Tyr Thr Lys Glu Thr Leu Asp Met Gln Glu Thr Pro Ser	
20 25 30	
cgc aag gtg gcc tcc gcc ttc atc atc att tta tgc tgt gcc atc gtg	144
Arg Lys Val Ala Ser Ala Phe Ile Ile Ile Leu Cys Cys Ala Ile Val	
35 40 45	
gtg gag aac ctt ctg gtg cta atc gca gtg gcc agg aac agc aag ttc	192
Val Glu Asn Leu Leu Val Leu Ile Ala Val Ala Arg Asn Ser Lys Phe	
50 55 60	
cac tca gcc atg tac ctg ttc ctc ggc aac ctg gca gcc tcc gac ctg	240
His Ser Ala Met Tyr Leu Phe Leu Gly Asn Leu Ala Ala Ser Asp Leu	
65 70 75 80	
ctg gca ggc gtg gcc ttc gtg gcc aac acc ttg ctc tcc gga cct gtc	288
Leu Ala Gly Val Phe Val Ala Asn Thr Leu Leu Ser Gly Pro Val	
85 90 95	
acc ctg tcc tta act ccc ttg cag tgg ttt gcc cga gag ggt tca gcc	336
Thr Leu Ser Leu Thr Pro Leu Gln Trp Phe Ala Arg Glu Gly Ser Ala	
100 105 110	
ttc atc acg ctc tct gcc tcg gtc ttc agc ctc ctg gcc att gcc atc	384
Phe Ile Thr Leu Ser Ala Ser Val Phe Ser Leu Leu Ala Ile Ala Ile	
115 120 125	
gag aga caa gtg gcc atc gcc aag gtc aag ctc tac ggc agt gac aaa	432
Glu Arg Gln Val Ala Ile Ala Lys Val Lys Leu Tyr Gly Ser Asp Lys	
130 135 140	
agc tgt cga atg ttg atg ctc att ggg gcc tct tgg ctg ata tcg ctg	480
Ser Cys Arg Met Leu Met Leu Ile Gly Ala Ser Trp Leu Ile Ser Leu	
145 150 155 160	
att ctg ggt ggc ttg ccc atc ctg ggc tgg aat tgt ctg gac cat ctg	528
Ile Leu Gly Gly Leu Pro Ile Leu Gly Trp Asn Cys Leu Asp His Leu	
165 170 175	
gag gct tgc tcc act gtg ctg ccc ctc tat gct aag cac tat gtg ctc	576
Glu Ala Cys Ser Thr Val Leu Pro Leu Tyr Ala Lys His Tyr Val Leu	
180 185 190	
tgc gtg gtc acc atc ttc tct gtc atc tta ctg gct atc gtg gcc ttg	624
Cys Val Val Thr Ile Phe Ser Val Ile Leu Leu Ala Ile Val Ala Leu	
195 200 205	
tac gtc cga atc tac ttc gta gtc cgc tca agc cat gcg gac gtt gct	672
Tyr Val Arg Ile Tyr Phe Val Val Arg Ser Ser His Ala Asp Val Ala	
210 215 220	
ggt cct cag acg ctg gcc ctg ctc aag aca gtc acc atc gta ctg ggt	720
Gly Pro Gln Thr Leu Ala Leu Leu Lys Thr Val Thr Ile Val Leu Gly	
225 230 235 240	

gtt ttc atc atc tgc tgg ctg ccg gct ttt agc atc ctt ctc tta gac 768  
 Val Phe Ile Ile Cys Trp Leu Pro Ala Phe Ser Ile Leu Leu Leu Asp 255  
 245 250

tct acc tgt ccc gtc cgg gcc tgt cct gtc ctc tac aaa gcc cat tat 816  
 Ser Thr Cys Pro Val Arg Ala Cys Pro Val Leu Tyr Lys Ala His Tyr 270  
 260 265

ttc ttt gcc ttc gcc acc ctc aac tct ctg ctc aac cct gtc atc tat 864  
 Phe Phe Ala Phe Ala Thr Leu Asn Ser Leu Leu Asn Pro Val Ile Tyr 285  
 275 280

aca tgg cgt agc cgg gac ctt cgg agg gag gta ctg agg ccc ctg ctg 912  
 Thr Trp Arg Ser Arg Asp Leu Arg Arg Glu Val Leu Arg Pro Leu Leu 300  
 290 295

tgc tgg cgg cag ggg aag gga gca aca ggg cgc aga ggt ggg aac cct 960  
 Cys Trp Arg Gln Gly Lys Gly Ala Thr Gly Arg Arg Gly Gly Asn Pro 320  
 305 310 315

ggt cac cga ctc ctg ccc ctc cgc agc tcc agc tcc ctg gag aga ggc 1008  
 Gly His Arg Leu Leu Pro Leu Arg Ser Ser Ser Ser Leu Glu Arg Gly 335  
 325 330

ttg cat atg cct aca tcg cca aca ttt ctg gag ggc aac aca gtg gtc 1056  
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tga 1059

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 <213> Murinae gen. sp.

<220>  
 <221> CDS  
 <222> (1)..(1119)

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aat gtg agc aac act ttg gac cct ggg gac acc cca ctg tct gca ccg 96  
 Asn Val Ser Asn Thr Leu Asp Pro Gly Asp Thr Pro Leu Ser Ala Pro 30  
 20 25

ctc agg atc tcg ctg gca ata atg atg ctg ctg atg atc gtg gta gga 144  
 Leu Arg Ile Ser Leu Ala Ile Met Met Leu Leu Met Ile Val Val Gly 45  
 35 40

ttc ctt ggc aac acg gtg gtc tgc atc atc gtg tac cag agg cca gcc 192  
 Phe Leu Gly Asn Thr Val Val Cys Ile Ile Val Tyr Gln Arg Pro Ala 60  
 50 55

atg cgt tca gct atc aac ctg ctg ctg gcc acc ttg gcc ttc tcc gac 240  
 Met Arg Ser Ala Ile Asn Leu Leu Leu Ala Thr Leu Ala Phe Ser Asp 80  
 65 70 75

atc atg ctg tct tta tgc tgc atg cca ttc acg gcc atc acc ctc atc	288
Ile Met Leu Ser Leu Cys Cys Met Pro Phe Thr Ala Ile Thr Leu Ile	
85 90 95	
act gtt cgc tgg cat ttc ggg gac cac ttt tgt cgg ctt tca gct act	336
Thr Val Arg Trp His Phe Gly Asp His Phe Cys Arg Leu Ser Ala Thr	
100 105 110	
ctc tat tgg ttt ttt gtc cta gag ggc gtg gcc atc ctg ctc atc att	384
Leu Tyr Trp Phe Phe Val Leu Glu Gly Val Ala Ile Leu Leu Ile Ile	
115 120 125	
agc gtg gac cgg ttt ctc atc atc gtg cag cgt cag gac aag ctg aac	432
Ser Val Asp Arg Phe Leu Ile Ile Val Gln Arg Gln Asp Lys Leu Asn	
130 135 140	
cca cgc agg gct aag atg atc atc gcg gcc tcc tgg gtg ctg tct ttc	480
Pro Arg Arg Ala Lys Met Ile Ile Ala Ala Ser Trp Val Leu Ser Phe	
145 150 155 160	
tgc atc tct gcg ccc tcc ttc act ggc tgg acg ttc atg gag gtg cct	528
Cys Ile Ser Ala Pro Ser Phe Thr Gly Trp Thr Phe Met Glu Val Pro	
165 170 175	
gct cga gcc cca cag tgc gtg cta ggc tac act gag ttc cca gct gaa	576
Ala Arg Ala Pro Gln Cys Val Leu Gly Tyr Thr Glu Phe Pro Ala Glu	
180 185 190	
cgc gcc tat gta gtg aca ctg gtg gtg gca gtg ttc ttt gct ccc ttc	624
Arg Ala Val Val Thr Leu Val Val Ala Val Phe Phe Ala Pro Phe	
195 200 205	
ggc gtc atg ttg tgc tcc tat ctg tgc atc ctc aat acg gtg cgg aag	672
Gly Val Met Leu Cys Ser Tyr Leu Cys Ile Leu Asn Thr Val Arg Lys	
210 215 220	
aac gct gtc cgt gtg cac aac cag tgc gac agc ctg gac ctc aga cag	720
Asn Ala Val Arg Val His Asn Gln Ser Asp Ser Leu Asp Leu Arg Gln	
225 230 235 240	
ctg acc gga gct ggc ctg aga cgt ctc aga cgg cag cag cag cag gcc	768
Leu Thr Gly Ala Gly Leu Arg Arg Leu Arg Arg Gln Gln Gln Gln Ala	
245 250 255	
agc ctg gac ctg agt ttc aaa acc aag gcc ttc acc acc atc ctc atc	816
Ser Leu Asp Leu Ser Phe Lys Thr Lys Ala Phe Thr Thr Ile Leu Ile	
260 265 270	
ctc ttc gtg ggc ttt tca ctc tgc tgg ctg cca cac tca gtc tac agc	864
Leu Phe Val Gly Phe Ser Leu Cys Trp Leu Pro His Ser Val Tyr Ser	
275 280 285	
ctg ctg tct gcg ttc agc cgg cgg ttc tat tac agc gcc tcc ttc tac	912
Leu Leu Ser Ala Phe Ser Arg Arg Phe Tyr Tyr Ser Ala Ser Phe Tyr	
290 295 300	
acc acc agc aca tgc gtc ctg tgg ctc agt tac ctc aag tct gtt ttc	960
Thr Thr Ser Thr Cys Val Leu Trp Leu Ser Tyr Leu Lys Ser Val Phe	
305 310 315 320	
aac ccc atc gtc tac tgc tgg agg atc aag aaa ttc cgc gag gcc tgc	1008
Asn Pro Ile Val Tyr Cys Trp Arg Ile Lys Lys Phe Arg Glu Ala Cys	
325 330 335	

ata gag ttg ctt ccc cac act ttc caa atc ctc cct aaa gtg cct gag 1056  
 Ile Glu Leu Leu Pro His Thr Phe Gln Ile Leu Pro Lys Val Pro Glu  
                   340                                  345                                  350

cgg atc cag agg aaa atc cag cca agc acc atc tat gtg tgc aac gaa 1104  
 Arg Ile Gln Arg Lys Ile Gln Pro Ser Thr Ile Tyr Val Cys Asn Glu  
                   355                                  360                                  365

aac caa tcc gct gtc tag  
 Asn Gln Ser Ala Val 1122  
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<212> PRT

<213> Mus sp.

<400> 9

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Asn Val Ser Asn Thr Leu Asp Pro Gly Asp Thr Pro Leu Ser Ala Pro  
                   20                                  25                                  30

Leu Arg Ile Ser Leu Ala Ile Met Met Leu Leu Met Ile Val Val Gly  
                   35                                  40                                  45

Phe Leu Gly Asn Thr Val Val Cys Ile Ile Val Tyr Gln Arg Pro Ala  
                   50                                  55                                  60

Met Arg Ser Ala Ile Asn Leu Leu Leu Ala Thr Leu Ala Phe Ser Asp  
                   65                                  70                                  75                                  80

Ile Met Leu Ser Leu Cys Cys Met Pro Phe Thr Ala Ile Thr Leu Ile  
                                   85                                  90                                  95

Thr Val Arg Trp His Phe Gly Asp His Phe Cys Arg Leu Ser Ala Thr  
                   100                                  105                                  110

Leu Tyr Trp Phe Phe Val Leu Glu Gly Val Ala Ile Leu Leu Ile Ile  
                   115                                  120                                  125

Ser Val Asp Arg Phe Leu Ile Ile Val Gln Arg Gln Asp Lys Leu Asn  
                   130                                  135                                  140

Pro Arg Arg Ala Lys Met Ile Ile Ala Ala Ser Trp Val Leu Ser Phe  
                   145                                  150                                  155                                  160

Cys Ile Ser Ala Pro Ser Phe Thr Gly Trp Thr Phe Met Glu Val Pro  
                                   165                                  170                                  175

Ala Arg Ala Pro Gln Cys Val Leu Gly Tyr Thr Glu Phe Pro Ala Glu  
                   180                                  185                                  190

Arg Ala Tyr Val Val Thr Leu Val Val Ala Val Phe Phe Ala Pro Phe  
                   195                                  200                                  205

Gly Val Met Leu Cys Ser Tyr Leu Cys Ile Leu Asn Thr Val Arg Lys  
                   210                                  215                                  220

Asn Ala Val Arg Val His Asn Gln Ser Asp Ser Leu Asp Leu Arg Gln  
 225 230 235 240  
 Leu Thr Gly Ala Gly Leu Arg Arg Leu Arg Arg Gln Gln Gln Gln Ala  
 245 250 255  
 Ser Leu Asp Leu Ser Phe Lys Thr Lys Ala Phe Thr Thr Ile Leu Ile  
 260 265 270  
 Leu Phe Val Gly Phe Ser Leu Cys Trp Leu Pro His Ser Val Tyr Ser  
 275 280 285  
 Leu Leu Ser Ala Phe Ser Arg Arg Phe Tyr Tyr Ser Ala Ser Phe Tyr  
 290 295 300  
 Thr Thr Ser Thr Cys Val Leu Trp Leu Ser Tyr Leu Lys Ser Val Phe  
 305 310 315 320  
 Asn Pro Ile Val Tyr Cys Trp Arg Ile Lys Lys Phe Arg Glu Ala Cys  
 325 330 335  
 Ile Glu Leu Leu Pro His Thr Phe Gln Ile Leu Pro Lys Val Pro Glu  
 340 345 350  
 Arg Ile Gln Arg Lys Ile Gln Pro Ser Thr Ile Tyr Val Cys Asn Glu  
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 370

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35

<210> 11  
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 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: primer

<400> 11  
 gcgcggtcga cggaagaaga gttgacgttt cc

32

<210> 12  
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 <212> DNA  
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<220>  
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<210> 13  
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<212> DNA  
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<220>  
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<400> 13  
gcgcgggtcga ctacagttgca gaagatccca ttc 33

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<212> DNA  
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<212> DNA  
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<210> 16  
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<212> DNA  
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gcgcgggatc caccatgggc ggtttatact cagag 35

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<212> DNA  
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<210> 18  
<211> 36  
<212> DNA  
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<223> Description of Artificial Sequence: primer

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gcgctctaga ccaccatggc ctgtaacagc acaccc

36

<210> 19  
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<212> DNA  
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gcgcgtcgcac ctagacagcg gattggtttt cg

32